

**THE DETECTION OF VIRAL ANTIGEN IN THE CEREBROSPINAL FLUID
OF PATIENTS WITH HERPES SIMPLEX VIRUS ENCEPHALITIS**

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**Thesis submitted for the degree of
DOCTOR OF MEDICINE
of the
UNIVERSITY OF EDINBURGH**

1990



ABSTRACT

A highly sensitive capture sandwich antibody assay was developed for the detection of Herpes Simplex Virus alkaline nuclease in cerebrospinal fluid. Chemiluminescence, using Isoluminol labelled antibody and p-Iodophenol enhanced Horse-Radish Peroxidase catalysed Luminol, was compared with Horse-Radish Peroxidase colorimetric detection in the assay. Luminol was superior to Isoluminol based chemiluminescence, but this was no more sensitive than colorimetric detection. In vitro the final optimised colorimetric assay had a sensitivity of 20 plaque forming units of Herpes Simplex Virus.

A method for dissociation of immune complexed alkaline nuclease, using Hydrochloric acid as a chaotropic agent, was optimised for use with the assay.

The assay was applied to 12 cerebrospinal fluid samples from 9 cases of Herpes Simplex Virus Encephalitis. All cases conformed to accepted criteria for the diagnosis of the disease in the absence of a brain biopsy. All the tested samples were positive for alkaline nuclease, which was present in either free or immune complexed form or both. The earliest positive sample was day 1 of the clinical illness, and samples were positive up to day 56, which was the longest interval from onset of symptoms to lumbar puncture in these patients. Free alkaline nuclease was present in the early stages of the disease, being replaced by immune complexed alkaline nuclease after day 13, a time when locally synthesised anti-HSV1 IgG was also detected.

A total of 35 control patients were also studied. Five out of 6 patients with suspected Herpes Simplex Virus Encephalitis or myelitis, and one case of definite herpes myelitis were positive. Two of the the remaining 28 cases were positive: both of these were patients with Subacute Sclerosing Panencephalitis. No demyelinating, inflammatory, other infectious or non-neurological disorders were positive by the assay.

ACKNOWLEDGEMENTS

My thanks go to Dr. Ed Thompson, who conceived the idea, supervised the work and encouraged me throughout the project.

Also to Dr. Geoff Keir, and Mr. Richard Luxton without whose technical advice and scientific support this work would have foundered early on.

To June Smalley, whose help with the manuscript and experience with word processing was invaluable.

To Dr. Bob Hoeness, Dr. Sandy Buchan, Dr. Ken Powell and Dr. Dave Meredith who generously gifted me antibodies and viral preparations, and also gave freely of advice on technical matters.

Finally, and most especially, to my wife, who endured a newborn baby and my thesis writing simultaneously without a murmur, and who has made all this effort worthwhile.

DECLARATION

The composition of this thesis, and the work described therein, was entirely the result of the authors' own efforts. (General regulation 1.2.7)

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SECTION 1

INTRODUCTION

CHAPTER 1: HUMAN HERPES SIMPLEX VIRUS ENCEPHALITIS

1. Introduction and Historical Review

A. Introduction

Herpes Simplex Virus Encephalitis (HSVE) is a rare complication of an infection with an extremely common virus. Despite its rarity, establishing the diagnosis has become increasingly important for two reasons. Firstly HSVE is the commonest fatal sporadic encephalitis in the developed western countries, survivors of the encephalitis having an exceptionally high morbidity. Secondly, there is a specific anti-viral agent available, Acyclovir, which is extremely efficient and successful at preventing viral replication. Thus of all the encephalitides of viral aetiology, those due to Herpes viruses, being the only potentially treatable disorders, become the most important in establishing a firm diagnosis.

As will become apparent in this review, the major problem in the diagnosis of HSVE is establishing the cause at the earliest possible stage in the disease to allow institution of effective anti-viral therapy. The criteria for establishing a diagnosis however, are the subject of continual debate, with views polarised between the USA and Europe, the former demanding brain biopsy and the latter advocating indirect methods of diagnosis. In this country, brain biopsy is generally not considered now to be an ethical procedure for diagnostic purposes in HSVE, therefore reliance on indirect methods, combined with clinical suspicions, are the main-stay of diagnosis. It was the need, therefore, for a reliable method of non-invasive and rapid diagnosis, that prompted the development of an assay for Herpes Simplex antigens in the cerebrospinal fluid (CSF) of encephalitis patients, which forms the basis of this research project.

Neonatal HSVE has not been specifically included within this review for two reasons. Firstly it presents as a separate clinical problem, with, in the main, a different Herpes Simplex virus to adult encephalitis, and a distinct pathogenesis and clinical picture. Secondly, the patients studied in this work were all adults with Herpes Simplex Type 1 Encephalitis. So saying, there will undoubtedly be included material from, and reference to, work performed using neonatal material, but this will not be specifically alluded to unless it is considered important to make a distinction.

B. Historical Review

The results of human infections with Herpes Simplex viruses affecting the skin have been reported for almost 2,000 years [256]. Hippocrates was familiar with herpes febrilis and shingles, but called only shingles by the term herpes. Herpes meant 'to creep', and was applied by the Greeks to diseases such as cancer, infections, ringworm and eczema. Herpes febrilis was known by a different name. The first English reference to herpes came in 1676, where Cooke commented

'In herpes there's little Pustules like to millet-seeds, Heat Itching, after rubbing a little moistness and little ulcers...' [46].

Probably the first notion of the modern concept of herpes was made by Bateman in 1814 in his book on cutaneous diseases, but he did not consider it infectious, and still included ringworm and erythema multiforme within the terminology [19]. Vidal, in 1873, first showed herpes to be infectious by human inoculation [265], and Gruber in 1920 demonstrated that herpes could be transmitted from animals to man, by the transfer of herpes from an infected rabbit to the cornea of a blind man [96]. The identification of the causative agents of cutaneous herpes, including cold sores and genital herpes rapidly followed, and it was not long after this that the first reports of a possible association with acute encephalitis were published by the Matheson Commission, in 1929 [165]. In this

survey, a literature review yielded 9 cases of epidemic encephalitis from which a herpes simplex virus was identified. Later work again suggested an association [88]. However, proof of the presence of herpes virus in brain tissue from patients with encephalitis remained elusive until 1941, when a report was published of a one month old child which died of a rapidly progressive diffuse encephalitis and intranuclear inclusions were identified in brain tissue and herpes simplex virus was isolated [236]. Once doubt about the association between herpes virus isolation and acute encephalitis had been removed, there ensued a rapid increase in literature reports and a realisation that other variously named acute encephalidites, such as acute haemorrhagic leukoencephalitis of Hurst [164], acute necrotising haemorrhagic encephalitis [37], acute necrotising encephalitis, acute inclusion encephalitis, acute necrotising encephalitis with inclusions, acute inclusion encephalitis of the herpes simplex type, acute inclusion body encephalitis, were all likely due to herpes simplex [21, 65, 3, 4]. Subsequently there was harmonisation of all these descriptions to a single diagnosis of Herpes Simplex Virus Encephalitis. In 1971, a literature review demonstrated that of 39 cases of acute necrotising encephalitis where electron microscopy was performed, 36 showed herpes simplex particles [207]. This finally confirmed that acute necrotising encephalitis and HSVE were synonymous.

Once the characteristic pathology of temporal lobe involvement was highlighted in the 1940's and 1950's [103], it was not long before attempts at diagnostic biopsy in life were made. Although the first biopsy report in 1956 did not confirm herpes simplex as the particular viral cause [63], in 1964 the first cases were confirmed by viral isolation [162]. Thus, the clinical spectrum of neurological disease caused by herpes simplex virus could be more widely identified to include not only those whose condition was fatal but also those who survived. Over the past 25 years, the clinical characteristics of the disorder have become well documented (for reviews see Illis et al [107], Whitley et al [278]), diagnostic criteria set down [80], and then specific treatment become available [206, 191, 113], such that now HSVE is the most important non-epidemic acute viral encephalitis affecting man.

In recent years, with the rapid increase in patients infected with the Human Immunodeficiency Virus 1 (HIV1) virus, opportunistic infection with herpes simplex viruses have presented a great diagnostic problem. It is, as yet, still too early to compare the disease with non-immunocompromised patients, and insufficient data is available to comment specifically on this group in great detail, but there is no doubt that in the future the recognised spectrum of HSVE may well change.

2. The Clinical Picture

A. Epidemiology

i) Incidence

Infection with herpes simplex virus type 1 (HSV1) is ubiquitous. By the age of 15, 50% of the population has antibodies to HSV1, and 90% by adult life [113]. Herpes simplex virus encephalitis (HSVE), by comparison, is relatively rare. In the United States 1,000-2,000 cases are estimated to occur each year [113], which accounts for 5%-10% of all annual reported encephalitis cases [101, 83], or an incidence of 1:250,000 to 1:500,000 persons per year [273]. In Sweden, the reported incidence is similar, at 2.3 cases per million inhabitants per year [233].

Neonatal HSV infection, usually HSV II type and acquired from infected maternal genital secretions at birth, occurs in about 1:2,500 to 1:5,000 deliveries per year in the United States and 50% of neonates so infected will have brain involvement [273].

In the United States, encephalitis in childhood is far more frequent than encephalitis in adults, with a peak incidence of about 30 per 100,000 of the population versus 5 per 100,000 [22], although herpes simplex virus is a commoner cause of encephalitis in adults than in children. This whole population based study found that in only a minority of cases could an aetiological agent be identified, but in those where a firm diagnosis was possible, 14% of those due to viral agents resulted from HSV infections. Hospital based studies of encephalitis give a similar picture. Even though the majority of patients with encephalitis have a causative agent identified (72%), the proportion found due to HSV remains the same at 13% of diagnosed cases [176]. HSV is also a cause of aseptic

meningitis, but is much less prevalent, accounting for only 2% of hospital cases where causal agents are identified. Combining aseptic meningitis and encephalitis, HSV accounted for 4.7% of all hospital diagnosed cases.

In Finland the reported annual incidence of hospital based encephalitis in children aged 1 month to 16 years has changed from 11.4 per 100,000 in 1968 to 3.4 per 100,000 in 1987, with an overall mean annual incidence of 8.3 per 100,000, of which 6.4% were due to HSV [138]. This dramatic reduction in encephalitis has been due to the introduction of successful vaccination programs against mumps, measles and rubella, and although the absolute incidence of HSVE has remained stable, the relative proportion has consequently increased.

In the United Kingdom data on the annual incidence of encephalitis, and particular specific encephalitides, is far more difficult to ascertain, because of the relative inefficiency of reporting and collection of data. One hospital based study over a 15 year period, found only 12 out of 60 patients with encephalitis had a confirmed diagnosis made, of which 6 were HSV. Of all cases of acute viral encephalitis admitted to the hospital during this time HSVE accounted for 10%. The problem of reporting of HSVE was confirmed in the USA in a full population based study from Minnesota [22], where the incidence of encephalitis was found to be 12 times that reported to the Centre for Diseases Control.

Table 1 shows the annual reported cases of all viral encephalitis and meningitis in the UK from 1978 to 1982, from all age groups. The figures suggest an annual incidence of between 1.7 and 3.3 per 100,000 population, of which HSV infections comprise between 6% and 11.4% (average 8.5%). When only those cases of HSV infections where there was unequivocal evidence of causal association between the virus and the encephalitis (i.e. viral isolation from brain, CSF, or raised antibody titres in CSF and/or serum) are used however, and cases of meningitis are excluded, the proportion drops to between

	1978	1979	1980	1981	1982	TOTAL
Echoviruses	680	308	778	386	298	2450
Mumps	479	260	182	240	108	1269
<i>Herpes Simplex</i>	103	98	107	109	95	512
Coxsackie B	136	69	74	171	51	501
Coxsackie A	25	56	30	33	71	215
Adenovirus	46	32	52	35	39	204
Measles	44	32	46	30	23	175
Varicella Zoster	43	25	39	30	32	169
Influenza B	1	23	-	3	30	57
Influenza A	13	8	15	6	8	50
Others	96	118	89	74	77	454
TOTAL	1666	1029	1412	1117	832	6056

TABLE 1 : Annual cases of viral meningitis and encephalitis reported to the Communicable Diseases Surveillance Centre 1978-1982¹⁹⁵ .

2.2% and 5.8% (average 3.7%), as shown in Table 2. Although the figures for incidence of encephalitis/meningitis are much lower than reported elsewhere, the proportion attributed to herpes simplex virus appears to be similar to the other studies. Thus in the UK one should expect to see from the official figures from the USA, Sweden and Finland, about 100 - 150 new cases per year, against the observed figures above of 37 - 52.

	All reports	Definite Association	Encephalitis only
1978	103 (6.0)	45 (2.7)	37 (2.2)
1979	98 (9.5)	47 (4.6)	40 (3.9)
1980	107 (7.5)	61 (4.3)	52 (3.7)
1981	109 (9.8)	59 (5.2)	47 (4.2)
1982	95 (11.4)	55 (6.6)	48 (5.8)
TOTAL	512 (8.5)	267 (4.4)	224 (3.7)

TABLE 2 : Annual reports of Herpes Simplex Virus associated encephalitis and meningitis 1978-1982¹⁹⁵ . (Percentages in parentheses).

ii) Sex distribution

From 805 cases in the literature where sex was reported, 443 (55%) were male 362 (45%) were female, a ratio of 1.22:1 male to female. This ratio has remained relatively constant over the last 20 years (Table 3), with only slight male to female preponderance throughout.

	Male	Female	M/F Ratio	Refs
1965	33	19	1.74	154
1972	107	81	1.32	107
1973	10	11	0.91	215
1982	61	52	1.17	278
1984	27	26	1.04	233
1985a	140	125	1.12	195
1986	43	26	1.04	274
1988	10	14	0.71	125
1989b	12	8	1.50	132
TOTAL	443	362	1.22	

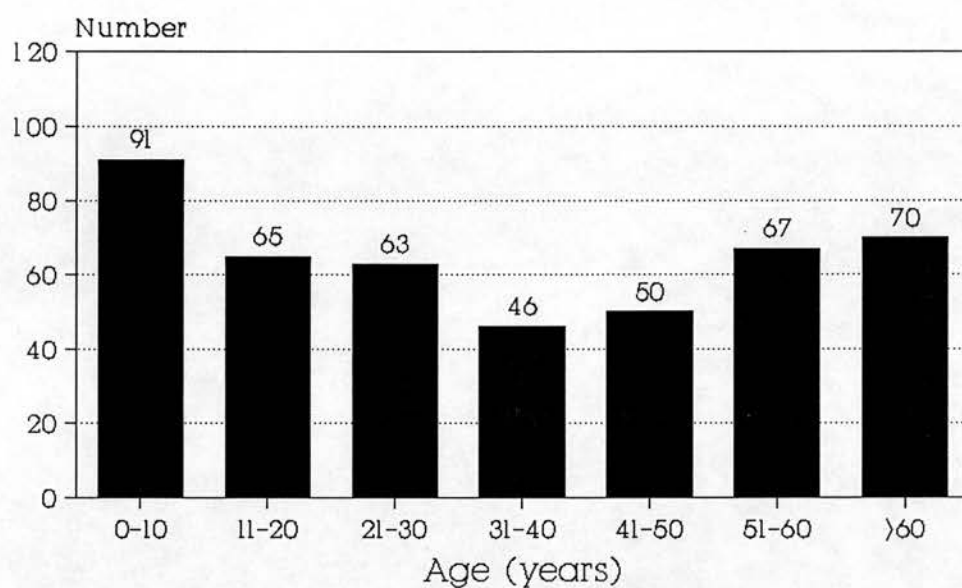
a - includes some meningitis cases

b - under 16 years old only

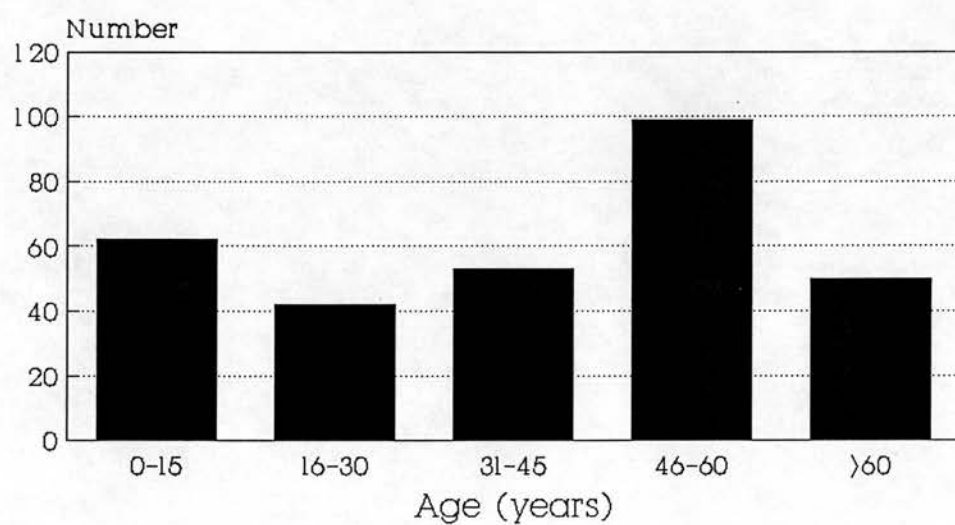
TABLE 3: Sex distribution of Herpes Simplex Virus Encephalitis in the largest reported series over the previous 25 years.

iii) Age distribution

A summary of the 8 major reports on HSVE shows that age distribution tends to bias towards the young and the old, but is not so striking as has been described by some (Figure 1). Two studies excluded patients under 6 months old, so that neonatal encephalitis, usually due to HSV2 and presenting with a different clinical pattern, were not included in their results.



a) Refs 107, 154, 215, 278, 274, 125



b) Refs 233, 195
 Figure 1 : Age distribution for cases of
 Herpes Simplex Virus Encephalitis

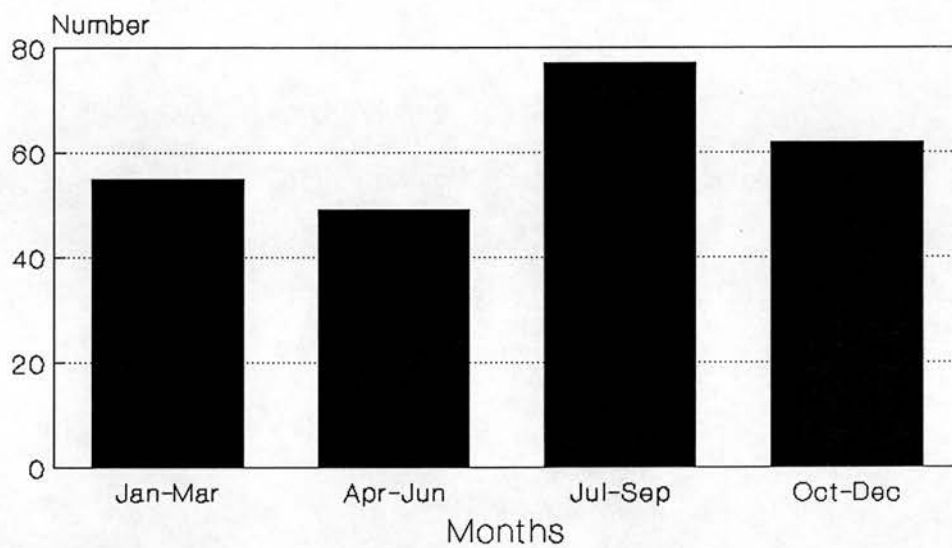


Figure 2 : Seasonal incidence of Herpes Simplex Virus Encephalitis

iv) Seasonal variation

Few studies have recorded any seasonal figures, and when known there appears to be no obvious seasonal association [154, 278, 107, 176, 193] (Figure 2).

B. Clinical Features

With the wealth of literature documenting the clinical course of both treated and untreated HSVE, a basic pattern of evolution is apparent which applies to most patients [80], but the variation in temporal aspects, severity and the occasional unusual and misleading presentations, make the spectrum of clinical features fairly wide.

There is usually a prodrome that can last for several weeks, often taking the form of a 'flu-like illness, but this can be absent in some with fulminant onset [125, 3]. In almost all patients, headache and fever and meningism are the earliest features of imminent neurological deterioration. Table 4 shows a summary of the clinical features from 442 cases, of whom 374 were biopsy proven, reported in the literature since 1965; before this time biopsy proof was often not available.

The majority had fever and headache on presentation (85% and 70% respectively), rising to 95% with fever during the illness. Meningism, including vomiting, occurred in about 40% of the patients. Consciousness was frequently altered at admission, with 68% drowsy, confused or disorientated, and 33% in coma. The state of consciousness related to timing of admission, and invariably worsened during admission with 40% in coma at some time. Personality change was frequent at presentation, occurring in 73% of patients rising to 88% after admission, and was often bizarre, with behavioural abnormalities mistaken for acute psychoses. Amnesia was reported in 22% of patients.

Clinically evident epilepsy was not as frequent as would be expected considering the degree of electroencephalography (EEG) abnormalities reported (see Chapter 2). 56% presented with epilepsy, with 70% having epilepsy at some stage in the illness. When recorded, focal epilepsy was the usual manifestation, and presented in 44% of patients, often in Jacksonian form. Considering typical EEG appearances, this is not surprising.

Year	Number (Biopsy positive)	Altered consciousness				Focal neurological signs					Seizures		Ref.
		Confused	Personality change	Memory loss	Coma	Hemi- paresis	Dysphasia	Cranial nn deficits	Field loss	Unspe- cified	All	Focal	
1965	13 (13)	7			6	2	4		1		6		154
1967	36 (21)	0 [12]				5 [17]	5 [7]				14 [23]	6	193
1970	11 (9)	[8/10]			[11]	[7/10]					[8/10]		207
1973	22 (21)	11	[8/10]			22	8					17	3
1973	16 (13)	10	11				4						194
1975	9 (6)		10		2					8 [9]	5		30
1977	28 (28)	27	19	5	[4]	12	14				16	4	276
1977	5 (5)				5						5		38
1978	13 (12)	12									8		73
1978	9 (9)	8	1			4	1 [4]		2		7	5	54
1978	15 (15)	12 [13]	7 [14]		1 [12]	2 [14]	1 [12]				6 [13]		280
1980	6 (3)	5			[4]						0 [3]		133
1982	113(113)		69/81	14/59	[7]	41/107	58/76 [19]	34/105	8/58		73/109	33/43	278
1984	53 (22)	53	46			[21]					33		233
1986	69 (69)												274
1988	24 (15)	14			10	15	13			19	11	7	125
TOTAL	442(374)	159/235 [33/61]	163/224 [22/25]	19/87	24/73 [38/94]	105/254 [59/114]	104/226 [42/113]	34/105	10/67	27/40 [9/9]	184/327 [101/145]	72/162	

[] - numbers at any time in disorder.

/ - figure following refers to patient number when it differs from total in study.

TABLE 4: Literature review of clinical features of biopsy proven series' of Herpes Simplex Virus Encephalitis.

a) Specific neurological features.

Year	Number (Biopsy Positive)	Fever	Headache	Vomiting	Viral Syndrome	Meningism	Papill- oedema	Ataxia	Autonomic dysfunction	Recurrent cutaneous Herpes	Refs
1965	13 (13)	13	4		8	3				4	154
1967	36 (21)	21 [33]	17 [19]	6 [9]	25	3 [20]				4*	193
1970	11 (9)	[10]	[8]			[9]					207
1973	22 (21)		11			21	12				3
1973	16 (13)		16			9				1	194
1975	9 (6)									6/27	30
1977	28 (28)	27	23	14				8/12	21/28		276
1977	5 (5)	5									38
1978	13 (12)	12	8		7						73
1978	9 (9)	8	4		4						54
1978	15 (15)	15 [15]	12 [9]	7 [8]	3		1 [1]			4*	280
1980	6 (3)	5	5	3		4 [5]	0 [3]				133
1982	113(113)	101/112	89/110	51/111				22/55	53/88	24/108	278
1984	53 (22)	53	39	20							233
1986	69 (69)	[67]	[46]								274
1988	24 (15)	19	13			9	3				125
TOTAL	442(374)	267/314 [125/131]	241/345 [82/131]	101/249 [17/51]	47/86	49/126 [36/71]	16/67 [4/21]	30/67	74/116	43/208	

* - indicates current herpetic cutaneous infection.

[] - numbers at any time in disorder.

/ - figure following refers to patient number when it differs from total in study.

TABLE 4 : Literature review of clinical features of biopsy proven series of Herpes Simplex Virus Encephalitis.
b) General features.

Only 19% of patients had generalised epilepsy alone, and 9% experienced both forms. In some patients, the seizures were manifested early in the disease by olfactory phenomena [94], or gustatory and gastrointestinal symptoms, highlighting the temporal lobe nature of the pathology.

Focal neurological signs of an unspecified nature were reported in 68% of patients on presentation rising to 100% during admission. When itemised, hemiparesis was present in 41% of patients on presentation increasing to 52% during admission, and speech disturbances, such as dysphasia and aphasia presented in 46%. In some patients, the aphasia was so severe as to mimic an akinetic-mute syndrome [3]. Cranial nerve deficits were found in 32% of patients, and visual field losses in 15%.

Other major features included autonomic dysfunction in 64% of patients, and ataxia in 45% of patients, both presumably related to brain stem involvement. Raised intracranial pressure, as manifested by papilloedema, was found overall in 24% of patients upon presentation, although in 1 study 55% showed raised intracranial pressure, a figure achieved in another small study only some time after admission to hospital.

In a typical patient, the illness falls into 3 phases [80]. During the first phase, meningitic symptoms predominate for the first 2-4 days, and is characterised by headache, fever and vomiting. This is followed by the second or local encephalitis phase from about days 3-7, where the temporal lobe features encountered above predominate, accounting for the seizures, speech and behavioral disturbance, and visual symptoms. As the pathology extends, the third or generalised phase with cerebral oedema takes over from about days 7-12. During this time the hemiparetic features, papilloedema, autonomic dysfunction, ataxia, and disordered consciousness predominate, ultimately in some resulting in death. Those who do not die usually show signs of slow recovery from about 2 weeks onwards.

The literature abounds with cases of atypical presentation of HSVE, although few substantiate the diagnosis with viral isolation. Only some of the more important and interesting variations will be considered in detail here.

The cases presenting with behavioral disturbances were highlighted by the papers of Drachman and Adams [66] who described 1 patient who:

"during the next night, he arose, dressed, shaved, awakened his wife, and declared that he was going to a funeral".

Subsequently, his illness resembled delirium tremens with

"conversations with imaginary people....., reached for imaginary objects, and picked at the bedclothes. A fine tremor of the outstretched hands and lips was present".

Psychotic symptoms could be so florid that patients were admitted to mental hospitals before the diagnosis became apparent [4].

Brain stem involvement was first reported by Dyanne et al 1972; [58], with 2 cases, 1 biopsy proven, which was not confined to the brain stem. Other cases have been reported since; 1 biopsy proven with a lateral medullary syndrome only [213], another 3 without biopsy evidence, 2 with focal brain stem signs, 1 of which presented with sudden respiratory arrest [82, 127] (also a feature of the case of lateral medullary syndrome above), and after recovering required sleep ventilation, simulating the syndrome known as "Ondine's Curse". Brain stem involvement has also been reported presenting as an isolated III nerve palsy [227], oculogyric crisis, trismus, masticatory and facial spasms [167, 82] and "locked-in" syndrome [167, 150, 143], but despite the severity of symptoms some patients have recovered completely [167].

A chronic encephalopathy, lasting more than 5 months has been reported in 2 patients, both positive for HSV isolation [217]. In one the patient had been treated for multiple sclerosis (MS), when at the age of 26, she developed a monoparesis, headache and

malaise, and had delayed visual evoked potentials (VEP's). However, chronic progression led to a revision of the diagnosis, but it was only the EEG showing bitemporal foci that led to a brain biopsy confirming HSVE. In the second, a 76 year old woman became forgetful and confused 3 months before admission. EEG and computed tomographic scanning (CT) were unhelpful, suggesting only encephalopathy, but serology suggested HSVE and the CSF cultured HSV isolates.

Not all forms of HSVE present with severe symptoms and signs. There has now been a report of 3 cases, 2 biopsy proven, where the clinical course was mild [128]. One patient developed only headache and confusion, 1 meningism, drowsiness and memory loss, and the third beginning with focal signs progressing to coma improved and then subsequently relapsed with meningism and coma. All 3 recovered without specific antiviral therapy, and only 1 patient was left with any sequelae, taking the form of grand mal epilepsy, although this latter patient had undergone brain biopsy. The suggestion was made that many more patients with mild forms of encephalitis may have HSVE, and that the incidence is thus underestimated, but more importantly, appropriate treatment may be withheld.

There have been reports of relapsing forms of HSVE. Ross and Stevenson [214] reported a 14 month old boy who, after an initial classical presentation, recovered completely only to relapse 30 days later with acute hemiparesis, hemianopia and chorieform movements. Recovery followed, but was not complete the second time. Shearer et al [229] reported a 9 year old boy whose recurrent herpes labialis (culture proven) were followed within 2-9 days by severe psychotic episodes on 17 separate occasions, with occasional focal neurological signs. With each episode, mental function slowly became progressively impaired. At the time of the reports there was great debate as to whether these were genuine relapses, with the first being termed by some critics a biphasic illness, and the second had no virological proof that the encephalitis was due to HSV invasion. Since this time however, undoubted relapses of HSVE have become well

documented. In several cases, relapse has occurred within days of discontinuation of specific anti-viral therapy which had resulted in complete recovery [260, 216], but in most the delay after successful treatment and relapse was longer at between 10 days and 1 month [233, 62, 56, 16], without any clinical doubt the relapse was virally caused. Relapse has been reported after treatment in other studies [97, 232, 133] but without pathological confirmation and with response to further therapy. In one study, relapses 14 months apart without specific treatment were confirmed by raised titres in serum to HSV and abnormal brain biopsy but without viral isolation [179]. Apart from the single case of Shearer reported above, there has been no documented case of more than one clinically defined relapse in the literature, so cases of chronic relapsing encephalitis or encephalomyelitis are unlikely to be due to HSV.

One extremely unusual complication of HSVE has been reported by 2 groups: Post HSVE encephalomyelitis or leukoencephalomyelitis. One case followed a classical "locked-in" brain stem syndrome, and at post-mortem, extensive demyelination of hemispheres and cerebellum were found, with HSV1 in the brain stem, especially the trigeminal area [143]. The second followed successful treatment of biopsy proven HSVE with adenine arabinoside (ara-A), and after what was thought to be a relapse, a further biopsy showed extensive demyelination with no evidence of viral reactivation. This raises the possibility that some cases labelled as relapses without biopsy evidence could be post infectious demyelination and this complication can certainly confuse the clinical picture. In the early studies of acute hemorrhagic leukoencephalitis, demyelination was in the context of massive necrosis, and was accompanied by Cowdry A inclusions, suggesting primary herpetic infection [164] rather than some form of autoimmune phenomenon, which the first 2 cases presumably represent.

Finally, the clinical spectrum of HSVE will be further complicated as the incidence of cases amongst HIV1 infected patients increases. The presentation is often in the context of multiple other pathologies, and making a clinical diagnosis can be difficult [149]. Thus in the future, the importance of non-clinically based diagnosis will be paramount.

C. Morbidity and Mortality

The advent of specific anti-viral therapy has altered the outcome for HSVE considerably. In this section, the course of the disease untreated with specific anti-viral therapy will be considered, although the number of series concerned is small, and not surprisingly the early literature paints a gloomy picture and generally does not dwell on the subject. The course of treated patients, and the comparison with untreated, will be dealt with in Chapter 3.

The problems of establishing a clinical diagnosis have resulted in many of the early studies focussing on fulminant cases, reporting post-mortem findings only, before biopsy in life was successful in 1963 [162]. Even after this time, case reports contained few numbers, so establishing the typical outcome was difficult.

Early reviewers of morbidity and mortality not only referred to each other, but also duplicated each others data and referred to common data [107, 250], so the morbidity and mortality figures are difficult to ascertain. Many early studies were flawed by lack of consistent diagnostic criteria, with some requiring brain biopsy results [193] and some relying solely on raised serum titres [214]. Consequently (and not surprisingly) those requiring brain biopsy had higher mortalities and morbidities than those whose diagnosis rested on serum titres alone. For example Rawls in 1966 [208] had 100% mortality in biopsy defined cases but Ross in 1961 [214] had only 13% mortality with 50% complete recovery in cases diagnosed on serological ground only.

With the diagnosis and reporting of encephalitis improving, the full impact of the severity of untreated HSVE became apparent. The pioneering epidemiological study of all encephalitides and aseptic meningitis referred to the Veteran's Administrations Hospitals over 5 year by Meyer et al in 1960 [176] found that although HSVE was not the commonest encephalitis, accounting for only 13% of all proven cases, with mumps

and lymphocytic choriomeningitis (LCM) being more frequent, the mortality untreated was by far the highest, with 5/13 cases dead (38%), 6 with severe sequelae (46%) and only 2 recovering to a "normal" state.

This finding was paralleled by the Communicable Diseases Surveillance Centre reports of encephalitis/meningitis in the United Kingdom from 1978-1982 (some 20 years later) where, although presumably most cases were treated with anti-viral agents, HSVE still had the highest mortality, with 60 deaths in 267 cases (22%), even though echovirus, mumps and coxsackie virus infections were all more frequently reported than HSV. Even though these statistics can be criticised, a review of all cases of encephalitis from a single hospital in London in 1981 [124] found that of 60 cases, in only 12 could an identifiable agent be found, 6 of which were HSVE (50%) and in which mortality was the highest at 4/6 (67%).

In the latest review of encephalitis from Finland in 1989 [132] HSVE had the highest mortality at 10%, and morbidity at 40% of all encephalitides caused by a single viral agent. Most, if not all, of these cases would have been treated with anti-viral agents.

Since the early reviews, most studies have concentrated on comparisons of therapies, initially with placebo, but then when that was deemed unethical, with other anti-viral agents. Therefore, most recent figures are culled from these patients, and added to the early non-treated summaries. A review of the literature reports of mortality and morbidity in untreated patients, where the diagnosis was made by brain biopsy or unequivocal serum and/or CSF antibody changes, is summarised in Table 5.

Of 160 patients, 94 (59%) died. Of the survivors, 17 (11%) suffered severe sequelae, 7 (4%) moderate and 25 (15%) mild or no sequelae, with 14 (9%) unspecified, and 3 (2%) not followed up.

Year	Patient Number	Dead	Sequelae in survivors				Ref.
			Severe	Moderate	Mild	None	
1960	13	5	6			2	176
1961	8	1	2	1		4	214
1965	13	3		4	2	4	154
1966	20	10	*	*	*	*	177
1966	5	5					208
1967	36a	25	5			3	193
1973	1	1					156
1973	8	7			1		3
1973	10	7		1	2		194
1975	1	1					30
1975	4	4					727
1977	10	7	1	1	1		276
1978	2		*	*	*	*	73
1980	1	1					133
1981	4	2				2	124
1982	10	6	3			1	69
1984	3				1	2	128
1988	11	9	*	*	*	*	125
TOTAL	160	94	17	7	7	15	

* - individual figures not given. Total 14 patients.

a - includes 3 survivors not followed up.

TABLE 5: Outcome of untreated Herpes Simplex Virus Encephalitis - literature review.

Thus only 15% of patients returned to a near normal state. The definition of mild or normal in some papers left a lot to be desired. In one review, 12 patients were said to be normal and then identified as containing 7 dysphasic patients. Many others claim a return to home and work as normality, but there is no doubt that psychological testing reveals subtle intellectual changes, and in some marked personality changes.

Mortality figures can be underestimated if follow up is for short periods of time. The majority of cases who succumb do so in the first 3 weeks after admission. Illis [107] gives figures of 94% of 99 fatal cases dying in the first 3 weeks with the remainder dying by the 6th week. However, later reports show patients dying up to 4 years after the encephalitis, of what was considered direct sequelae of the disease [194, 276].

Because of the numbers of survivors in the untreated patients was relatively small, few generalisations can be made, and most studies did not differentiate between treated and untreated survivors. Oxbury et al [194] did detail the outcome in untreated patients. All severe sequelae survivors had varying degrees of dementia, from mild to severe with behavioural problems. Dysphasia was common especially of the jargon variety. One patient had a severe amnesic syndrome, of the type typically associated with bilateral hippocampal lesions, and 2 patients displayed signs similar to Kluver-Bucy syndrome with unrestrained eating, disinhibition, and marked oralisation. Half of the severely affected patients were tetraparetic, and also complicated by epilepsy. Of untreated mild sequelae survivors, mild dysphasia, personality change, memory and learning deficits predominated.

Some untreated survivors did return completely to normal, and in some, coma was present at some stage in the illness [128], but usually only mild cases recovered so well. Signs and symptoms could continue to improve for months to years after the illness, sometimes even after a delay of months. Age at presentation did not have any influence on mortality, except in under 2 months group, which in one review had 100% mortality against 50% - 83% for each other decade of life [107]. Later studies however, suggested that the very old and the very young did consistently worse than middle years, though these were tied in with therapeutic trials with anti-viral agents, and so will be considered later. Other prognostic features will also be considered later under their individual headings.

3. Laboratory Findings in HSVE

A. CSF Abnormalities

CSF changes reflect the viral aetiology of the process, and the necrotic nature of the pathology. Since the disease undergoes a rapid evolution, CSF changes also evolve, and this variability results in the conflicting comments made by investigators, who were not necessarily comparing patients at the same stage of disease. Table 6 shows the main CSF findings reported in the major series'.

The majority of patients show an elevated total protein, up to 7.88 g/l, with white cell counts of up to 1,400/mm³, which are predominately lymphocytes. Although polymorphonuclear leucocytes can be present at some stages, this is usually a minor component even though one author reports them at up to 90% of the total WBC count [193]. The presence of red blood cells (RBC) has caused most confusion, since small numbers of cells may reflect traumatic taps rather than true haemorrhage, and one report emphasises that absence of xanthochromia in patients [97]. Most studies confirm the presence of RBC's, up to 7,000/mm³, but usually below 500/mm³. The CSF glucose is usually normal, although a few patients have a CSF:serum ratio below 50%. (Occasionally elevated values are given, without mention of the serum values).

When intracranial pressure measurements have been recorded, an increase is reported in 28 of the 64 cases (44%). In one series, sequential measurements showed a progressive rise in intracranial pressure, peaking around 2 weeks after the disease onset, and those patients with higher pressures initially were more likely to die [15].

The CSF may be initially entirely normal, and may remain normal throughout [97], but this does not necessarily indicated mild disease [194].

Year	Number Patients	Protein (mg/dL)		Glucose (mg/dL)		Pressure (cm H ₂ O)		WBC (/mm ³)		Lymphs. (/mm ³)		Polymorphs (/mm ³)		RBC (/mm ³)		Normal Initial Profile	Ref.
		Range	No.	Range	No.	Range	No.	Range	No.	Range	No.	Range	No.	Range	No.		
1965	6		5		1			12-1400		Most	3	0-25		50-178	4	0	37
1966	5	35-516	4	50-118	0	18-30	2	0-700		13-756	5	0-90%				4	208
1967	36	15-258	34	67-110		7-29	3/3									4	193
1972	5	32-165	4	<45	2	7-33	4	1-800		74-100%						1	112
1973	16	90-240	6					62-950	10	60-900	10	6-220		Unusual		4	194
1973	12							2-100	21	Most		Unusual		Unusual			59
1973	22		19				12									3	73
1978	13		10							9-1212	11	<17%		20-7060	8		133
1980	6	30-788	6	<50%*	1			0-1040		>83%							
1982	98	7-755		<50%*	5			0-1100									
		<40	18					0-5	4					0	16	3	
		40-100	43					6-50	18					1-50	37		
		>100	37					51-500	68					51-500	23		
								>500	8					>500	18		278
1984	6							0-325		>63%		<11%					245
1985	53							20-300	49	31-246				6-255	5	7	232
1987	8		1		0												97
TOTAL	276	15-788	169/215	50-118	9/128	18-33	28/65	0-1400	174/189	9-1212	26/39	0-220	11/21	0-7060	104/136	19/168	

No. - refers to numbers with increased values, except for glucose, where numbers are decreased values.
 * - % of serum glucose value.

TABLE 6: Routine Cerebrospinal Fluid abnormalities reported in the major studies in the literature.

In those studies where sequential punctures have been performed, the initial profile earliest in the disease may be normal, with the total protein most often elevated early [133], gradually rising to a maximum within 2 months, then slowly declining. Cells become apparent within the first 4 days [245], again peaking within the first few months, then declining. The initial profile often shows some polymorphonuclear leucocytes and macrophages, but this soon shifts to almost entirely lymphoid populations [133, 245, 193], which are invariably pathologically enlarged stimulated cells after day 12 [245].

This profile is by no means diagnostic of HSVE [225], with non HSVE diseases having similar abnormalities. In a comparative study of brain biopsy positive and negative patients, only the elevated WBC count showed a statistically significant difference in the HSVE patients [278].

A few other proteins have been studied in the CSF in HSVE. Ferritin and S100 levels, markers of haemorrhage and astrocyte damage respectively, have been found to be elevated in the CSF from the 3rd day, persisting to at least 40 days [231], and not in other encephalitides, TB meningitis or neurosyphilis. These proteins are not specific for HSVE, occurring in other traumatic and haemorrhagic disorders.

B. Immunoglobulin changes

Before the advent of sensitive assays, immunoglobulin studies were limited to the examination of changes in serum only. Neutralisation and complement fixation tests for specific IgG showed that accompanying the neurological disease there was an elevation of the serum titres up to very high levels, although there was rarely any detectable response before day 5, and not all patients showed an increase [215].

30% patients are seronegative at the onset of the clinical disease, seroconverting during the course of the illness, indicating that the infection is primary. 70% of patients have detectable titres at onset, showing a rise during the illness, indicating secondary reactivation or reinfection. Up to 7% of patients, however, fail to demonstrate any significant increase in serum titres [188].

Current serological testing has provided much greater sensitivity, and changes in serum and CSF antibody levels, both total and HSV specific, are now well documented. Total IgG, and locally synthesised IgG as measured by the IgG index, have been demonstrated to be elevated in the CSF from day 10 onwards, and to remain elevated for over a year in some patients [133, 134, 245]. In the same patients, serum and CSF HSV specific titres are elevated, with ratios of serum to CSF of 32:1 to 1:1, indicating local synthesis. Elevated CSF titres have sometimes been found as early as day 4 [188], but usually not before day 8, but they may remain elevated for over a year [133, 111] and up to 29 months [134, 245].

The most sensitive techniques, measuring quantitatively specific IgG responses, confirm that the serum response is usually only detectable around day 10, but with a rapid increase over a few days to a high level, falling slowly thereafter over months. In some patients the rise is much slower and the levels achieved less dramatic. CSF changes parallel the serum changes, and the levels as a proportion of the total IgG are always in excess of the serum proportions [81].

Studies of specific IgM responses are much less common, as sensitive assays only became available in the 1980's. Serum specific IgM titres have been reported as early as day 2 and 3 [34, 126], and elevated CSF titres with local synthesis by days 5-6 [34, 245, 231], remaining elevated for about a month before declining thereafter [231].

Total IgA has been found to be locally synthesised in CSF after day 5 [231, 245, 263], remaining elevated for about a month, as with IgM, and then declining after [231]. Animal models have also confirmed this finding [148]. Specific IgA responses have only been reported in one paper, and although it was found in serum and CSF from day 2, the poor presentation of the data meant that local synthesis of specific IgA could only be shown from day 16.

Anti HSV1 specific IgG subclass reactivity has been studied in serum and CSF in two studies [111, 166]. Both found IgG1 predominating, with IgG3 and IgG4 less common and IgG2 least often found. One group found the serum and CSF distribution to be identical [111]; the other group reported local synthesis of all subgroups, particularly IgG1 which was usually the earliest response, although in some patients the development of IgG3 and IgG4 local synthesis could precede IgG1.

There have been no reported studies of the specificity of the response in HSVE in humans to particular viral epitopes. Animal models appear to show a difference in the specificity of the IgG response to primary and secondary infection, noting that in primary infection the anti-glycoprotein activity is too late to contribute to viral clearance [172, 174]. The IgM response also differs in epitope specificity to IgG and in primary and secondary infection [140]. A mouse model of HSVE has also demonstrated the presence of anti-myelin basic protein antibodies, but the relevance of such antibodies to the pathological changes is uncertain [25].

The role of the immunoglobulin responses to HSV infection in the diagnosis of HSVE is considered in more detail in Chapter 2, where the qualitative aspects of the intrathecal immunoglobulin response i.e. oligoclonal banding, will also be discussed with respect to diagnosis.

C. Cellular responses

As indicated in section A, the cellular response is predominantly lymphoid, and precedes the antibody response [81]. The pleocytosis usually appears from day 4, peaking within 1-2 months, then declining, but may persist for up to 16 months. The neutrophil and plasma cell response is usually early, but the lymphoid response takes over, and the cells that display reactive features predominate from day 12 [245].

One study has examined lymphocyte sub-types in a case of HSVE. The lymphocytic cell surface markers show that whilst pan B cells predominate early, they decline to be replaced by cells with pan T markers. In addition, the T4:T8 ratio in the CSF parallels blood until about day 20 when the ratio increases only in CSF, declining to blood levels by 50 days, indicating compartmentalisation of the response [10]. Animal models have demonstrated high concentrations of T4 and T8 lymphocytes adjacent to HSV infected cells in areas of myelin loss, indicating that in animals, and perhaps in man, lymphocytic responses may be responsible for as much of the pathology as the virus itself [39].

CHAPTER 2: DIAGNOSIS OF HERPES SIMPLEX VIRUS ENCEPHALITIS

1. Diagnosis Using Clinical Criteria

A. Clinical Diagnosis

From the review of clinical features in Chapter 1, it can be seen that in the early stages, symptoms are non-specific, consisting of vague malaise, 'flu-like symptoms, headache, fever, mild meningitic or encephalitic symptoms, so clinical diagnosis is almost impossible at this stage. In only a minority of patients, about 20%, is there a history of recurrent or recent herpetic infection (see Table 4b), which, given the prevalence of HSV gingivo-stomatitis in the community, does not aid particularly in the diagnosis. In one comparative study, the incidence of recurrent HSV lesions was 24/108 in HSVE sufferers and 18/81 controls or 22% in both [278]. It is only when signs of focal temporal lobe involvement become apparent that the suspicion of HSV pathology becomes raised. Thus, any febrile patient with signs of disturbed consciousness or behavior, and focal neurological symptoms and signs, especially focal epilepsy, Wernicke's dysphasia or hemiparesis, should be considered to have HSVE. Even better if temporal lobe symptoms predominate, such as olfactory or visual hallucinations, or amnesia.

HSVE, however, is only one of several conditions that can cause fever, confusion, focal neurological signs and temporal lobe involvement. The difficulty in correctly identifying HSVE from clinical criteria, even when some supplementary investigations have been instituted, is illustrated by the results of the major trials into therapy for HSVE. In the Boston Inter Hospital (BIH) idoxuridine study in 1975 [30] 12 patients with clinical features indicative of focal encephalitis were recruited, space occupying lesions were excluded and other fungi and bacteria were not cultured from CSF, yet 3 did not have

HSVE on biopsy. In the 1981 National Institute for Allergies and Infectious Diseases (NIAID) collaborative study on vidarabine [277], which included for statistical purposes the 1977 study results [276], 182 patients were enrolled with a clinical diagnosis of HSVE, of which 76 (42%) did not have HSVE on biopsy. One could argue that biopsy was falsely negative, but in 35 (19%) of the 76 cases an alternative diagnosis became apparent, and in 37 of the remaining 41 cases brain histology suggested viral meningo-encephalitis due to agents other than HSVE. The remaining 4 had serological changes suggestive of HSVE. In the NIAID study of 1982 [278], which updated 1981 work, of 202 patients recruited, 113 were positive for HSVE and 89 (44%) were not HSVE on biopsy with 35 having alternative diagnosis, and in 50 other viruses likely. The collaborative Swedish Herpes Encephalitis Study Group in 1984 [233, 232] enrolled 127 cases with clinically suspected HSVE, of which 74 (58%) were considered finally not to have the disease, although not all were brain biopsied. 22 of these latter patients were found to have another cause for their disorder. Finally, in NIAID study of 1986 [274], of 208 enrolled patients undergoing brain biopsy, all again clinically diagnosed as suspected HSVE, 139 (67%) did not have HSVE on biopsy. Only 3 (2%) of these latter patients had serological changes compatible with HSVE.

Table 7 summarises the clinical diagnostic accuracy of these 5 major studies over 10 years, during a time when experience of clinicians in these centres should have improved the accuracy. As one can see however, the accuracy steadily declines from 75% in 1975, to 33% in 1986. This was partly attributed to the realisation of the larger spectrum of disease that existed, with the inclusion of milder cases, the lowered threshold for diagnosis, given that by 1986 acyclovir was in use, and the early referral of patients in an attempt to have therapy instituted, given the associated between conscious state and timing of therapy with outcome. Overall, clinical diagnosis was correct in 45% of patients, or less than half. This does not necessarily mean that clinical diagnosis correctly identifies all those patients who have HSVE; mild cases may survive

unrecognised, and in others the diagnosis may only be apparent post mortem, but aside from individual case reports it is impossible to assess how many false negatives clinical diagnosis does make.

Trial	Year	Patient Number	HSVE (%)	Non-HSVE (%)	Ref.
BIH	1975	12	9 (75)	3 (25)	30
NIAID	1982	202	113 (58)	76 (42)	278
SMS	1984	127	53 (42)	74 (58)	233
NIAID	1986	208	69 (33)	139 (67)	274
TOTAL		549	244 (44)	305 (56)	

BIH - Boston Inter Hospital study

NIAID - National Institute for Allergic and Infectious Diseases Collaborative study

SMS - Swedish Multicentre Study of Skoldenberg et al

TABLE 7: Accuracy of clinical diagnosis in major trials of therapy in Herpes Simplex Virus Encephalitis.

It would be unreasonable to suggest that many serious cases escape diagnosis in neurological centres, given the other means of aiding in diagnosis, but it would be interesting to see how many clinical diagnoses without investigative aids are revised. Whitley et al [278] estimated that in only 83% of patients had HSVE been clinically suspected (and this included the use of CT, EEG, but excluded biopsy and serology). The importance of accuracy in diagnosis is therefore not only to identify HSVE for antiviral therapy, but also to treat appropriately those conditions mimicking HSVE which may be eminently treatable.

B. Differential Diagnosis of Herpes Simplex Virus Encephalitis

Before considering the differential diagnosis of a clinically defined case, it should be pointed out that in practice many investigations now exist to exclude many of the alternative diagnoses, and with brain biopsy, the diagnosis of a positive, and one would hope a negative case should not be in doubt. But, as illustrated already, the early features of HSVE may be non-specific, and the first focal features suggestive of temporal lobe problems may arise with any temporal lobe pathology.

In the 1960's, it was apparent that "acute necrotising encephalitis" caused by HSV1, could present as an expanding space occupying lesion, with angiograms and air ventriculograms demonstrating shift of vessels [37, 4]. It was important to exclude tuberculous meningitis and meningo-encephalitis, or cerebral abscess, both treatable with specific therapy, and cerebral tumours, the symptoms of which could be relieved by decompression. In the monograph by Illis and Gostling on HSVE in 1972 [107], their table of differential diagnosis does not differ greatly from the diseases found to be clinically diagnosed as HSVE in recent studies (Table 8). Commonest confusing diagnoses were other viral meningitides which presented like HSVE, but tumours, tuberculosis and abscesses are still being misdiagnosed. Of the 35 patients (of 76 total) misdiagnosed in Whitley's study, 11 could potentially have been treated in some way, and in particular 7 would have required specific therapy, which would not have been instituted as soon as was done when brain biopsy was performed. In the Swedish study of Skoldenberg [233], 22/74 non HSVE patients were given an alternative diagnosis, of which 9 were potentially treatable and required specific therapy. There is obviously no confusion in some diagnostic categories now - multiple sclerosis is unlikely ever to be confused with HSVE.

	Illis 1972 ¹⁰⁷	Whitley 1981 ²⁷⁷	Skoldenberg 1984 ²³³
Viral	Post infectious Arthropod-borne Echovirus + others	Post influenza St. Louis encephalitis Coxsachie Mumps EBV SSPE LCM	Post influenza Tick-borne Mumps EBV Adenovirus Enterovirus Rubella CMV
Non-viral	Tumour Abscess Bacterial meningitis	Tumour Abscess Cryptococcus Toxoplasma	Citrobacter Listeria Mycoplasma
	TB meningitis SAH Subdural haematoma Multiple Sclerosis Degenerative encephalopathy	Tuberculosis Vascular disease	Vascular disease
2° effects	Metabolic disease Toxins and poisons Sarcoid Septicaemia Acute porphyria Infestations	Reyes syndrome Toxic encephalopathy	Alcoholic

TABLE 8 : Differential diagnosis of Herpes Simplex Encephalitis. 1972 - 1984.

More recent studies have focussed on individual cases presently like HSVE, but having other confirmed diagnoses. There have been 2 case reports of isolated small vessel angiitis of the brain, presenting in an identical fashion to HSVE. In the first [184] a 15 year old girl developed malaise, headache, confusion, followed by a generalised seizure and in 12 hours had a right hemiparesis, dysphasia and was drowsy. A CT brain scan showed a left parietal hypodense mass. A brain biopsy showed necrosis only and at post mortem only necrotising vasculitis was seen. In the second case [261], the signs and symptoms more closely mimiced the typical course of HSVE, with a 10 day prodrome of 'flu-like symptoms, then decreasing conscious state and left hemiparesis with dysphasia. Vidarabine was commenced before a biopsy showed angiitic changes with little or no parenchymal changes.

A review of cases of eastern equine encephalitis (EEE) from Massachusetts over 14 years [204] described the clinical similarity of the condition with HSVE. There was in general a similar vague prodromal period, most patients developed focal signs, such as cranial nerve palsies or hemiparesis, many were confused and drowsy, and many were treated as HSVE with appropriate antiviral agents. However, the EEG (non-specific background changes) and CT (generalised oedema in EEE) were found to be distinguishing features once the diagnosis was confirmed serologically. Until serological results were available, CT or EEG could not be used to unequivocally exclude HSVE.

A retrospective study of 25 acute viral encephalitis cases not caused by HSVE was used to examine what features, if any, could be used to exclude HSVE in early phase [225]. Using clinical, laboratory, radiological and electrophysiological features, the authors decided none were able to reliably exclude HSVE and 9 of these cases had other aetiological agents identifiable: 3 epstein-barr virus, 3 measles, 2 varicella, and 1 rubella. Five of these did however, have preceding exanthem which would have led to the correct diagnosis. In particular focal signs were appropriate to the temporo-parietal area clinically in 7, and on EEG in 13. Twelve patients were so clinically similar to HSVE that antiviral therapy was instituted. One major caveat was the lack of biopsy evidence to exclude HSVE, and the exclusion made on serological grounds. The authors admitted that

"... our series might contain patients with serologically negative HSVE".

The importance of being aware of potentially treatable other conditions when one does not use viral antigen detection as a means of diagnosis cannot be underestimated. Apparent response to therapy should not be taken as evidence of correct diagnosis, and failure to respond should prompt a re-evaluation of the diagnosis along the lines illustrated. One must also remember the unusual presentations of definite HSVE, illustrated in Chapter 1, which may initially suggest another diagnosis.

2. Diagnosis by Detection of Specific Antibody to Herpes Simplex Virus

A. Serological Diagnosis

By convention, proof of primary infection with an organism by serological means requires evidence of an increase in specific antibody production by a factor of at least 4-fold. To detect such a change, 7-10 days usually has to elapse between samples. In a disease of such rapid progression therefore, serological diagnosis is usually of little or no help in the acute management of the patient, and offers only retrospective diagnosis. So saying, in the time before brain biopsy in life was shown to be feasible, and in those countries which still do not accept brain biopsy as an ethically justifiable procedure (such as the United Kingdom), serological evidence is often the only laboratory supportive means of diagnosis.

Complement fixation tests (CFT) are the usual methods applied to HSVE, although neutralisation antibodies can be assayed to discriminate between HSV1 and 2 [207]. In early studies a 4-fold rise in serology was taken as definite evidence of HSVE, so many studies did not compare serology with brain biopsy in the same patients. In addition, brain biopsy was often a post mortem biopsy, and death occurred before a second serum sample was taken, so comparisons were often impossible.

In the first non fatal case undergoing brain biopsy in 1956 [63], the serology taken after day 15 showed a 4-fold decrease in CFT to HSV, taken as evidence of the aetiology. It wasn't until 1964 and the first 2 biopsy cases in life were reported, that both were shown to have greater than 4-fold rises in CFT [162]. Miller et al in 1966 [177] reported 20 cases, of which 10 were biopsy positive. Although 14 patients showed greater than a 4-fold rise by CFT, of the 10 patients who died only 4 had rising titres, with all having paired sera analysed. No patient exhibited a response to other viral agents, but they demonstrated that any primary infection i.e. focal herpes labialis or genitalis could result

in greater than 4-fold rises to high titres, and during quiescence titres were always below 64. Thus careful search for intercurrent mucocutaneous herpes was indicated in case the serological response was consequent upon this and not the encephalitis. Leider reported 15 encephalitis cases of which only 2 were virus positive. In one, considered HSVE on serological grounds, there was a similar 4-fold rise in influenza titres, so that accuracy of this diagnosis remains in doubt [154]. A similarly disappointing association was found in the study of Olson et al (1967) [193], who reported 15 cases of serologically diagnosed HSVE in whom 4 biopsy was attempted and were all negative.

Ross et al in 1973 [215] reported 20 cases, of whom 15 had virus identified. Eleven had >4-fold elevation in CFT titres, some from <8 to >1024, with 3 showing high static levels, and 5 dying before a second sample could be taken. Two biopsy negative patients were serologically positive. Four patients showed a significant elevation to herpes zoster by complement CFT, but this was not thought to be a problem as neutralisation tests could be used to differentiate.

Illis et al [107] suggest that in mucocutaneous herpes, serum titres never achieved the level seen in encephalitis. They reported 9 cases of mucocutaneous herpes with a highest CFT titre of 320, and the mean 93. For 6 encephalitis cases the highest titre was 2,560 and the mean 640. Neutralisation titres were only done in 3 cases of HSVE, but paralleled the CFT findings.

Rappel in 1970 [207] noted that the appearance of CFT antibodies in serum alone could not be taken as evidence of HSVE, as other diseases could reactivate CFT antibodies. Johnson [116] noted non-specific rises with other intercurrent infections such as pneumonia and hepatitis.



Because not all cases of biopsy proven HSVE have rising antibody titres in serum to HSV, and not all cases of rising antibody titres were associated with positive biopsy results, and in some patients there were simultaneous rises to more than one agent, reliance on serology alone has fallen from favour, until now in many centres it is not considered sufficient evidence to establish the diagnosis. Despite this, reports still appear of HSVE "proven" on serum antibody changes alone, and indeed the majority of cases in the United Kingdom of HSVE are still "confirmed" by serum antibody rises alone (168/267 reported to the PHLS) [195].

B. CSF Serology

Since Kabat first identified abnormalities of CSF immunoglobulins in neurological diseases in 1942 [117], it became apparent that specific encephalitides were associated with intrathecal production of specific antibody to the aetiological agent. Early studies produced initial poor results, with CSF neutralising and CFT antibodies not being detectable before day 9 at the earliest and usually not until after day 14 if at all [161, 113]. As assay sensitivities improved, it became apparent that antibody detection was feasible consistently in the CSF, but interpretation of the findings was sometimes mistaken because of the failure to appreciate that CSF immunoglobulins are also derived from serum by passive transfer.

In 1978 Levine et al [155] were the first to identify local synthesis of specific antibody within the CNS in biopsy proven cases by using both passive haemagglutination, and a more sensitive immune adherence haemagglutination assay on CSF and simultaneous serum samples, and using the ratio to establish evidence of local synthesis. They were aware that passive transfer of serum immunoglobulins to CSF would give positive CSF results if serum titres were high enough, so included a cut-off value of serum:CSF ≤ 20 before considering a result to indicate local synthesis. By these criteria, they established local synthesis in all 8 patients studied, 3 of which gave a positive result before brain biopsy was performed, the earliest at day 4 of the illness and in 4 patients by the 10th day of the illness. However, 4/28 controls were also positive: 1 HSV2 meningitis, 2 varicella zoster infections and 1 multiple sclerosis. The first 3 were readily explained by cross-reactivity, but the MS patient required invoking non-specific activation of CSF antibody. A more likely explanation was the failure in their analysis to correct for blood brain barrier damage, which could reduce the serum:CSF ratios in the absence of true local synthesis. Radioimmunoassay for specific IgG was initially reported in 1981 by 2

groups. Both used solid phases coated with antigen to capture specific antibody. Klapper et al [129] calculated an antibody index to correct for blood brain barrier damage:

$$\frac{\text{CSF HSV antibody}}{\text{Serum HSV antibody}} \times \frac{\text{Serum Albumin}}{\text{CSF albumin}}$$

Forty seven control patients with back pain were used to establish a cut off value of 1.91. They studied 26 patients with HSVE (14 biopsy or necropsy diagnosed), and found local synthesis of specific antibody in 24. Five of 10 patients studied before day 10 were positive, 3 of the initial negatives becoming positive after day 10. The earliest positive result was on day 3. However, no disease positive controls were included in the study, so the specificity of the assay could not be verified.

The second study by Skoldenberg et al [234] used polystyrene balls as solid phase, and studied 16 patients with HSVE, 4 of which had viral isolation from CSF, brain biopsy or necropsy. They similarly corrected for blood brain barrier damage by using albumin or a non-neurotrophic control virus (respiratory syncytial virus). They demonstrated local synthesis of IgA, IgG or IgM in 1/4 patients 3-4 days after disease onset, 5/9 patients 6-8 days after disease onset and in all patients after day 10. For controls, HSV2 meningitis were used, 4/5 showing IgM local synthesis, and 42 non-neurological patients, none of which were positive. In the HSVE patients, long term follow up showed local synthesis of IgM declining after 2-3 months, but in 2 patients persistence for 4 and 15 months was demonstrated. IgA findings paralleled IgM, with persistence in 4 patients beyond 7 and up to 32 months. Persistent IgG production was evident in all patients up to 43 months, maximal by 4 months, with slow decline thereafter.

Thus both RIA methods using single paired CSF and serum samples could detect locally synthesised antibody in all patients beyond the 10th day, but before day 10 were far less successful. Both studies omitted positive controls with non-herpetic neurological illnesses. Thus the crucial problem of early diagnosis to institute appropriate therapy still had not been resolved, and the specificity of the systems properly assessed.

Felgenhauer et al in 1982 [81] used a wide variety of positive controls, and a sepharose 4B solid phase colorimetric ELISA assay, to demonstrate local synthesis in all 25 cases of HSVE (12 biopsy proven) but only recorded 1 positive result before day 10 and did not correct for barrier damage. Koskiniemi et al [134] looked at 11 patients (6 biopsy proven) by CFT to a number of viruses, and calculated an IgG index to determine local synthesis of total IgG, but not specific IgG. All patients after day 10 demonstrated local synthesis of herpes specific IgG but only 7 had an elevated IgG index indicating locally synthesised antibody and absence of relevant data for the other 4 patients means local synthesis could not be verified in these. Only 2 patients had detectable CSF antibodies to any other virus, both varicella zoster. Forty-four control patients, including other encephalitides did not produce HSV specific locally synthesised antibody. Local synthesis of specific IgG in the HSVE patients was prolonged, up to 29 months, as found by Skoldenberg et al [234]. Nahmias et al (1982) [188] in their NIAID study, used the serum:CSF ratio of specific IgG and found 90% of 113 biopsy positive patients had local synthesis as defined by a value of ≤ 20 , but also 19% of 93 biopsy negative cases, although barrier damage was not corrected for. The majority however, did not show local synthesis until after day 10. In the 1984 Swedish multi-centre study [233], Skoldenberg et al reported 25/26 biopsy proven HSVE patients to have locally synthesised anti-HSV IgG with barrier damage corrected for, but noted that in many cases the response only occurred after day 12 of the illness. Tests for simultaneous rises to measles and varicella zoster, showed only a few patients demonstrated the latter and the rise was small. They found the test specific, with 24 patients with non-HSV or VZV infections, both bacterial and viral, not showing any response. However, some reports

have doubted this apparent specificity of response, with one report of mumps meningitis showing apparent local synthesis of anti-HSV IgG [262], and another presumed TB meningitis having local synthesis of anti-HSV IgG [44], although in this latter case there was no good evidence that HSVE could not have occurred since no tubercle bacilli were ever isolated.

Since these studies, other attempts have been made to improve the serological studies and their sensitivities. Jeansson et al (1983) [109] developed an in-house ELISA kit based on solubilised HSVE infected cell membranes containing all the major HSV glycoproteins and demonstrated intrathecal synthesis of specific IgG in 3 patients (without barrier correction but with measles virus as a control). The earliest response was day 10, but levels rose approaching serum levels for up to 7 years in one patient. Sugimoto et al [242] studied 3 children using a commercial ELISA kit and demonstrated specific IgG from day 3-4 in the CSF when conventional CFT and neutralisation tests were negative, and Keunen et al [126] used an ELISA to show local synthesis from day 16 of specific IgA in 2/5 patients, but neither studies used adequate correction for blood brain barrier function. Johansson et al [111] corrected for BBB damage in 10 patients (3 biopsy positive), found local synthesis in 9/10 but only 1/5 before day 10.

Using purified glycoprotein B as a captor antigen, Kahlon et al (in 1987) [118] demonstrated with a radioimmunoassay that 35/36 biopsy proven HSVE CSF samples contained specific antibody, but also 6/22 controls. They did not correct for BBB damage in any quantitative fashion, but used the presence of CSF antibodies to adenovirus as an indicator of damage. By this criteria the 6 controls could be excluded, but no mention was made of how many biopsy proven cases would have to be excluded. The earliest recorded day for a positive result was not given, but day 5 mentioned as the time after which most became positive.

Finally, intrathecal synthesis of IgG subclasses has been examined [111, 166], with IgG3 and IgG4 being the earliest intrathecally synthesised subclasses in some patients, although IgG1 was the commonest intrathecally synthesised antibody, with most producing at least 2-3 subclass types. No researchers were detecting antibody much earlier than day 10.

Thus intrathecal synthesis of specific antibody is sensitive (up to 96% of biopsy proven cases), in most studies is specific (although isolated case reports suggest occasional increases in other diseases), is non-invasive, but is virtually useless before day 10 of the illness, by which time the pathological damage has reached its peak and many patients are in coma. As a diagnostic tool, it is reserved therefore for making a retrospective diagnosis, although when positive before day 10, and as early as day 3, the results can be taken as confirming HSVE.

C. Oligoclonal Bands

Oligoclonal IgG, locally synthesised within the CNS, has been associated with CNS infections, including HSVE, since the phenomenon was first identified. In Skoldenberg's study of 11 patients [234] 4 of which were biopsy proven, locally synthesised oligoclonal IgG was not detected before day 10 in the 6 patients where it was sought, but was found in all after 10 days, and in every sample analysed during follow up, for up to 1,103 days. The specificity of the oligoclonal bands was investigated by Vandvik et al in 1982 [263] in 7 cases of HSVE, using imprint immunofixation of agarose electrophoresed or electrofocused IgG and autoradiography. All 7 patients had local synthesis of oligoclonal total IgG, but not before day 9, and all showed HSV-specific oligoclonal IgG, in 1 case on day 5 despite the total IgG being negative. HSV-specific oligoclonal IgG was visible up to day 390. Oligoclonal HSV-specific IgM was only found in 3/6 patients, between days 15 and 37, but HSV-specific IgA was found in all 6 patients tested, from day 8 up to day 390. The results were specific, in that normal patients, and patients with other neurological diseases, did not show any response to HSV. However, 1 HSVE case did show a transient small oligoclonal response to measles, suggestive of non-specific activation. The oligoclonal HSV-specific IgG was estimated to account for about 50% of the total CSF IgG in 1 case where elution was attempted.

Further refinements to the technique have confirmed the sensitivity and specificity of the method using, using isoelectric focusing antigen immunoblotting and HRP catalysed staining [182, 64], but further studies using antigen radioimmunoblots on purified glycoprotein B were flawed technically and results reported showing local synthesis in some patients were not convincing because of the excessive artefactual banding visible on the illustrations, and the interpretation of these were open to question [95].

Thus, like quantitative studies, qualitative studies were generally not positive until after day 9-10, and even though specific immunoblotting could detect HSV-specific IgG earlier than total oligoclonal IgG, the use of this technique for diagnosis was recognised as limited.

3. Diagnosis by Detection of Virus or Viral Antigens in CSF

A. Recovery of Virus

Without exception, the rate of recovery of HSV from the CSF is disappointingly low, and is thus one of the least useful means of establishing a diagnosis. There have been several individual case reports of HSVE with viral isolation from the CSF, the first being Armstrong et al in 1943 [7], who isolated the virus from a case initially suspected of having lymphocytic choriomeningitis (LCM), and over the years occasionally others have been reported, none of whom had brain biopsy proven disease [128, 150, 217].

Table 9 summarised the major studies where positive attempts have been made to isolated the virus from CSF, and the results given when mentioned.

Year	Patient Number	Isolation attempted	Isolation positive	Ref.
1961	5 (0)	5	0	214
1965	6 (6)	6	1*	37
1966	22 (22)	10	0	208**
1966	20 (10)	20	0	177
1967	36 (21)	7	1	193
1973	20 (14)	9	0	215
1973	17 (?)	17	1	251
1975	9 (9)	9	1	30
1977	28 (28)	28	1	276
1980	6 (3)	6	0	133
1982	113 (113)	45	2	188
1985	267 (63)	(?)	10	195
TOTAL		162a	7a	

() - numbers within are biopsy positive cases

* - probably ventricular fluid

** - review paper

a - excluding 1985 paper

TABLE 9: Literature review of results of attempted isolation of herpes simplex virus from the cerebrospinal fluid of patients with herpes simplex virus encephalitis.

Rawls study of 1966 [208] was a complete literature review of all cases from 1941-1966 where brain biopsy was positive, and there were no cases in 10 attempts where HSV was successfully isolated. Subsequent reports refer to therapeutic trials, or reviews of personal experience, so there was no doubt as to the attempts being made to culture the virus. Excluding the 1985 PHLS report, where the number of cases where attempts to culture were performed was not stated, of 162 attempts, most of which were biopsy positive, only 7 cases were successful. In one of these, the CSF was obtained after death and so was likely to be ventricular [37], which was also the source in a case of Klapper et al (not included in the table), when CSF was taken at biopsy [128]. Indeed Flewitt in 1973, who studied 12 biopsy proven patients states:

"we have never isolated herpes febrilis virus from lumbar CSF. Virus is usually present in ventricular CSF, though CSF often takes 1-2 days longer than brain suspensions...." [84].

In no cases has virus been isolated from lumbar CSF and not from brain tissue if biopsy has been performed.

Thus, even if one includes the PHLS study and assumes all had CSF culture attempted, only 4% of CSF will yield a positive result. This probably represents the highest figure, as innumerable studies don't even mention results of CSF viral culture presumably because of negative results.

B. Detection of Viral Antigens in CSF

Recovery of whole virions from CSF that are viable, can be cultured and then reidentified, is disappointing, but whether poor results are related to absence of virus, or that virus is present but either inactivated or modified or in insufficient quantities to be adequately cultured remains uncertain. Given the degree of brain necrosis in some patients, it would be surprising if virus was absent from CSF, so it is likely that it is either present in small quantities or modified or inactivated in some way.

Sommerville in 1966 first reported identification of HSV by immunofluorescence in cells from patients with herpes meningitis^[285], but no one was able to substantiate this finding [158]. Dayan in 1973 reported an immunofluorescent method for examination of the cells in CSF of patients with encephalitis for presence of viral antigens [59]. Ninety-five patients were examined, 33 with a variety of proven encephalitides, of which 87 they correctly identified as encephalitis or not encephalitis. Three patients were incorrectly identified as not encephalitis. Of 12 cases of HSVE (the diagnostic basis for this was not specified), 11 were positive for cells containing HSV antigens. In 2 cases of clinically progressive HSVE for 3 weeks, antigen containing cells were present for as long as the signs progressed. In 1 case, cells recovered within 24 hours of onset of symptoms were negative, but a second sample 30 hours later was positive. Numbers of positive cells was given as a percentage and was 10%-25% at best, but since absolute numbers of cells in each specimen was not given and the methods were incompletely reported, the significance of this finding was disputed [251, 159]. Indeed Longson and colleagues pointed out that in cases of positive culture from CSF, cells cannot be demonstrated containing viral antigen [159], and Nahmias et al found only 2/47 biopsy proven cases had immunofluorescent detection of antigen in CSF cells [188]. Since Dayan's report, no one has published confirmation of these findings, and when specifically looked for, no positive results have ever been found [133]. Since CSF cells

have thus proved an unreliable source, investigators have redirected their efforts to the detection of free viral antigen in CSF, although there have been comparatively few reports in the literature.

Chen et al in 1978 [41], purified and radio-iodinated a glycoprotein from HSV infected rabbit kidney cells, which although not fully characterised, appeared to be a structural non-envelope glycoprotein. Using a competitive assay, CSF's from 6 biopsy proven cases of HSVE were examined and all were found to block precipitation by between 15%-66% with no negative controls showing blocking (3 seizures, 1 mumps encephalitis). Although the authors mention that antigens were detectable early in the course of disease in CSF, no data was given. They also confirmed the problem of using a competitive assay when CSF samples would also contain specific antibody which could interfere. The rationale for use of an excreted glycoprotein was that in patients this was more likely to be present in extracellular fluid at an earlier stage than whole virions.

Coleman et al in 1983, developed a competitive inhibition ELISA for HSV antigens in CSF [45]. By coating a plate with HSV, incubating the CSF with a known amount of polyclonal rabbit anti-HSV globulin, and measuring the reduction in binding to the plate versus controls consisting of uninfected cells supernatants, they examined CSF's from neonates and adult HSVE patients, all biopsy proven. They found 61% of neonatal CSF's showed significant reduction and 15/23 (65%) of adults who were brain biopsy positive, but also 4/29 (14%) of brain biopsy negative controls were positive. They found no association between presence of HSV specific antibody in the CSF and the result, and fewer patients were positive early in the disease than later. (10/21 <10 days, 11/14 11-20 days, and 5/6 >20 days). They were unable to determine what epitopes the polyclonal antibody were directed against, but showed they were common to both HSV1 and 2.

Bos et al in 1987 developed an ELISA capture technique based on a sandwich assay principle [29]. Using microtitre plates as solid phase, CSF antigen was captured by polyclonal goat anti-HSV1 IgG and probed by polyclonal rabbit anti-HSV1 IgG, with a final amplifier of sheep anti-rabbit peroxidase conjugate and OPD substrate. The method was made more elaborate by the running in parallel of a control plate of preimmune goat IgG, so that an index could be calculated. By running HSV infected vero cell extracts and vero cell controls, they established a cut-off ratio of 1.5, modified to 1.93 when it was found there was some cross-reactivity of rabbit antisera with goat hyperimmune but not preimmune antisera. Eighteen patients with HSVE were examined, of which 5 were biopsy positive and 3 had CSF isolation of virus. Twelve controls consisted of non-infectious neurological disorders and 21 non-HSV encephalitis cases. The results were disappointing. Of 38 CSF's studied from the 18 patients, only 8 were positive, representing 6 patients (33%). Only 1 of the 3 patients with virus isolated from CSF produced a positive result. One of the 33 controls was positive, a case of encephalitis of unknown origin. In all 6 positive patients, the first CSF taken was positive, and in 1 case with 3 serial punctures the CSF remained positive from day 15 to day 19. When serial samples were followed, decreasing antigen ratios were sometimes accompanied by increasing levels of intrathecal synthesis of specific IgG, suggesting the possibility of immune complex formation binding antigen, but only 2 cases of positive antigen ratios had serial samples, neither of which supported this contention. The earliest day recorded for a positive result was day 5, and the latest day 19, but for 4 of the positive patients no dates of samples were given. The negative samples dated from day 2 to day 37. Although the sensitivity with this assay was poor at 33% and worse than intrathecal antibody detection, the specificity was good at 97% and antigen could be detected from day 5.

Agut et al in 1988, employed a commercial HSV1 antigen detection ELISA kit (Wellcome), which was developed for the diagnosis of genital herpes [6]. This simple capture ELISA probed by an NADPH linked monoclonal antibody was adapted by

prolonging the incubation of antigen to 20 hours. Twenty-one cases of HSVE and 24 non-herpetic infections were analysed and straight optical density results plotted. Nine of the twenty-one (43%) HSVE CSF's studies were above 3 standard deviations of the mean of the non-herpetic controls and also exceeded the highest value for the non-herpetic group. The highest OD values were obtained in those patients within one week of onset of clinical signs, and who had not yet any recorded anti-HSV1 IgG. The lowest OD's were recorded from those whose CSF's were taken after one week and with the presence of anti-HSV1 IgG. Overall 7/9 patients without specific IgG were positive for antigen and only 2/8 patients with antibody were positive. The sensitivity of the test, thus was only 43% but the specificity 100%. The results also suggested that immune complexing was occurring after the development of the antibody response, thus preventing antigen detection. The earliest time for a positive result however, was not given.

Lakeman et al in 1987 employed a pool of monoclonal antibodies to glycoproteins B, C, D and E to probe CSF's which had been dried onto nitrocellulose membranes [147]. The final antibody was anti-mouse, either radio-iodinated or biotinylated. Twenty-six biopsy positive and 17 biopsy negative patients were studied, using 40 and 25 CSF's respectively. Radiolabelled antibody give identical results to the streptavidin-biotin systems. Thirty-five out of forty biopsy positive specimens gave a positive result (88% specificity) and all 5 negatives being part of the 13 CSF's taken within 7 days of onset of the illness. Thereafter, all specimens were positive, including 3 from patients initially negative. Three of twenty-five biopsy-negative specimens gave positive results (88% specificity). Antigen could be detected up to one month after onset and as early as 3 days. Taking patients individually, the sensitivity was 92% (24/26) and the specificity 82% (14/17). Specimens taken within one week of onset of disease were only 64% sensitive, after this time rising to 100% sensitivity. This method was by far the most sensitive antigen detection method reported, but the results contrasted dramatically with those of Agut et al, who could not find antigen after day 7. The monoclonal probe used

by the commercial kit, is believed to be against glycoproteins B or C, so the result should have been similar. In this study the effect of native CSF IgG was found to be negligible. Perhaps the differential antibody response in CSF to glycoproteins may explain the results. Lakeman et al commented that the use of glycoprotein B monoclonal antibody alone gave poor results.

Viral DNA has been sought in CSF, using dot blot hybridisation techniques. Shuster et al in 1986, using in vitro synthesised radiolabelled RNA transcripts, examined CSF samples from 2 patients which had been both SDS and proteinase K treated before DNA extraction and spotting and drying onto nitrocellulose filters [226]. Both samples were positive for DNA: 1 patient had also HSV DNA detectable from post-mortem temporal lobe and the other was diagnosed on the basis of intrathecally synthesised IgG and IgA. Neither CSF's were culture positive. No negative controls were run, but the probe was demonstrated not to react with CMV or varicella zoster DNA and to only a small degree with HSV2 DNA. The assay sensitivity was 3 pg of plasmid DNA (1.5×10^5 genomes).

Boerman et al in 1987 used radiolabelled nick-translated DNA fragments for hybridisation to DNA extracted from CSF's from a single patient and dried onto nitrocellulose filters [28]. The diagnosis of HSVE was made on the basis of a 4-fold rise in CSF specific IgG titres, without reference to serum values. CSF culture again was negative. CSF's taken on day 12 and day 23 were both positive for specific DNA, the latter more so. The assay sensitivity was 1 pg of human HSV1 DNA (0.5×10^5 genomes).

Both these assays took under 48 hours, but the procedure was elaborate, reagents not readily available to most departments, and only 3 patients in total were reported, with no patient controls with other neurological disease. However results do show that DNA is present and for some considerable time in the CSF. Antibody complexing prevents viral detection in vitro but the detection of DNA is not affected.

Further evidence for the role of immune complexes has come from 2 studies using particle counting immunoassays demonstrating immune complexes in the CSF of patients with HSVE increasing from day 12 of the disease in parallel with intrathecal specific antibody production [230, 231]. Immune complexes persisted for 3-4 weeks then decreased, but specific antibody production was maintained thereafter.

Finally there has been one previous single attempt at applying chemiluminescence to herpes antigen detection. Pronovost et al in 1981 [203], developed a chemiluminescent immunoassay (CELIA) to HSV antigens based on a double antibody sandwich with rabbit polyclonal anti-HSV1 as captor and human anti-HSV1 as detector. The assay compared absorptiometry with luminescence in detection, with CELIA detecting 40 plaque forming units (PFU) versus absorptiometry 2,500 PFU. When clinical samples were run, the CELIA was successful in detecting HSV1 from 15/18 mucocutaneous lesions of both HSV1 and HSV2 sera types but was unable to detect any antigen from 6 CSF specimens which were also culture negative. No specific details were given of the CSF specimens so little further comment can be made except that the assay in these circumstances was completely unsuccessful.

Table 10 summarises all these studies, comparing their specificity and sensitivity. The detection of antigen in the CSF has been shown to be feasible in at least 7 separate studies (excluding Dayan's disputed work): 2 for generalised HSV1 antigens, 2 for DNA and 3 for envelope and non-enveloped glycoproteins. Radiolabelling and enzyme catalysed substrate reactions were both successful in either competitive or non competitive assays. However, the sensitivity varied considerably, from 33%-100%, and in some depended on the timing of the assay, with some evidence suggesting immune complexes may play an important role. Antigen was detected as early as day 3 of the illness, which is as early as the most optimistic of the antibody based diagnostic tests. The chemiluminescent assay however, was unsuccessful in CSF testing, despite the sensitivity that it appeared to confer.

Year	Assay	Antigens	Sensitivity	Specificity	Ref.
1973	Immunofluorescence	Cells	11/12 (92)	78/78 (100)	59
1978	Radio-immunoassay	Glycoprotein**	6/6 (100)	4/4 (100)	188
1981	Sandwich ELISA - chemiluminescence	All antigens	0/6 (0)	N.D.	203
1982	Immunofluorescence	Cells	2/47 (4)	N.A.	464
1983	Competitive ELISA	All antigens	15/23 (65)	25/29 (86)	45
1987	Sandwich ELISA - HRP/OPD	All antigens	6/18 (33)	32/33 (97)	29
1987	Solid phase Ag assay - avidin/biotin - radiolabelled	Glycoproteins B,C,D,E	24/26 (92)	14/17 (82)	147
1988	Sandwich ELISA* - NADPH	?Glycoprotein C	9/21 (43)	24/24 (100)	6

* - Commercial kit, antigen detected unconfirmed

** - antigen detected non-envelope structural glycoprotein

() - percentages

TABLE 10 : Sensitivity and specificity of assays for antigen detection in the cerebrospinal fluid of patients with Herpes Simplex Virus Encephalitis.

The principle of viral antigen detection has thus been established, but is not so successful that it can be considered an alternative to other methods. There is clear evidence that antigens may be present early enough in the CSF to warrant the pursuit of this avenue of research, which was the main aim of this study.

4. Diagnosis by EEG

The EEG tended to be ignored as a diagnostic aid in the earlier reports of HSVE.

Drachman and Adams in 1962 for example, only gave brief mention to the EEG in their 6 cases, reporting that although all were abnormal, none was distinctive, and only 1 showed focal features with sharp waves, the rest being diffuse slow wave activity predominantly lateralised [66]. The first detailed report to show significant changes in a series of HSVE cases came from Rawls et al in 1966, when in reporting 5 cases all postmortem proven, 4 of the EEG's all taken within the first 8 days of the neurological illness were markedly abnormal, with 3 showing localised sharp waves or spike and wave complexes at regular intervals which sometimes developed into focal seizures [203]. These had been traditionally associated with cerebral infarction and tuberculous meningitis, where the term "periodic lateralised epileptiform discharges" was applied.

Until this paper, the association between these focal discharges and encephalitis had not been recognised, and was concluded to be a reflection of the localisation of the severe pathological changes. Thereafter, despite the occasional report to the contrary [4], the value of the EEG in HSVE in demonstrating localised abnormalities and correlating with the pathological changes became increasingly recognised [193, 156]. Upton and Gumpert reviewing their own cases and some from the literature, summarised the typical findings in HSVE as being a diffuse slow wave background, periodic complexes from day 2-15 after the onset of the illness at about 0.5-1.0 Hz, which disappear thereafter, rapid onset of the complexes and disappearance irrespective of clinical state, and abnormal activity in frontotemporal regions, mainly periodic complexes and sharp and slow waves [257]. These, they felt were pathognomonic features. Illis and Taylor in 1972 in their review of the literature and their own 20 cases, reported similar findings, though were less stringent in their limitations, finding about half the patients had focal temporal lobe abnormalities with persistence of periodic complexes up to several weeks, and the degree and type of abnormality correlating more closely with the severity of the

illness and with timing [108]. They suggested their findings indicated localisation of pathology rather than any specific type of pathology and thus were not pathognomonic. Elian in 1975 extended this work by studying 11 cases and following up patients up to 4 years [72]. The presence of repetitive complexes was correlated with poor prognosis, in that all 7 with these died, and 4 without survived, although later work found bilateral changes gave worse prognosis than unilateral [34]. Chi'en et al in 1977 reported findings in support of Upton and Gumpert, in that a comparison of the pre-biopsy EEG's of 5 cases of biopsy proven HSVE and 12 biopsy negative encephalitis cases, showed 3/5 HSVE had distinct high voltage periodic sharp waves, around 0.3-0.5 Hz, from the temporal lobes with none of the controls showing these [38]. Furthermore, the EEG localised the best site for brain biopsy, with the highest viral yield from the temporal lobes showing the focus. These findings have been substantiated and extended since, and although the temporal aspects vary, the patterns have remained constant [133, 34, 69].

It became apparent that EEG changes could be present early on in the disease, in some cases focal abnormalities appearing from day 2 of the clinical neurological symptoms [34, 97]. When compared with other neurodiagnostic tools, such as CT (see later) the EEG was either the only abnormality or was abnormal earlier [69]. In many cases, the EEG is the first abnormality detected [97, 125]. In Whitley's major studies on HSVE, the EEG showed localisation more frequently than brain scanning, with 81% of patients with biopsy proven HSVE having focal EEG abnormalities, versus 50% of technecium brain scans and 59% of CT brain scans. 65% of proven cases showed the characteristics spike and slow wave abnormality localised to the area proven by biopsy. It was concluded that the EEG was the most useful neurodiagnostic evaluation, but that this was not specific for HSVE, with up to 59% of non HSVE patients showing focal EEG changes, the nature of which were unspecified.

Although this apparent non specificity was confirmed in another study, where 13/25 non HSVE patients showing focal temporal alteration, and only 4 showing periodic activity [225] in some diseases such as EEE the EEG characteristically does not contain periodic complexes, and can be used for differential diagnosis [204]. Overall the EEG sensitivity is good, but the specificity is poor in the early stages before periodic complexes appear, and even then are not pathognomonic. However, it is consistently the earliest investigative abnormality, suggesting that once neurological symptoms have appeared, pathological damage must be fairly severe.

5. Diagnosis by Neuroradiology

A. Angiography, Encephalography, Ventriculograms and Isotope Brain Scans

Because of the common presentation as a space occupying lesion, many early studies employed one or more combinations of these investigations with mixed success [208, 263, 37]. In the review of Illis and Gostling of radiological investigations available until 1972, 46% of patients overall were normal, and 41% showed a temporal abnormality [107]. Isotope brain scans and carotid arteriograms were better, at 47% and 57% respectively, than ventriculograms and air encephalograms at 16% and 17% respectively. Apart from localisation of the abnormality, they gave no indication of the pathology, and have now been superceded by more sophisticated radiological techniques.

B. Computed Tomography and Magnetic Resonance Imaging

The advent of computed tomographic scanning (CT) and subsequently magnetic resonance imaging (MRI), has improved the role of radiological imaging in HSVE, but not to the degree hoped. The first major study using CT scanning appeared in 1978 using 9 biopsy proven cases [54]. The earliest abnormality was seen to be a bilateral mass effect and abnormal contrast enhancement, with later low-absorption (attenuation) changes in the temporal lobes. There were no localising features on any of the 6 scans before day 5 of the neurological illness, with 4 being normal and only 2 having mass effect at 1 and 3 days. All scans were abnormal, including with contrast enhancement, by 7 days. A simultaneous report from another centre on 13 patients with HSVE showed 12 scans abnormal, with 10 having focal medial temporal or insular cortex low attenuation [73]. However, scans were taken later into the illness, with the earliest scan at 4 days. A comparison with cerebral angiography and radio-nucleotide imaging showed a good concordance, with 90% of both studies being positive. The one patient with an initially normal CT scan had an abnormal focal EEG. Three non HSVE patients used as controls showed no focal temporal abnormalities. Follow up studies in these patients showed worsening clinical states paralleled by evidence of increasing CT involvement with haemorrhage often in the form of linear streaking, which was considered characteristic. Progression on CT could last several weeks.

Other studies have confirmed the high incidence of focal CT abnormalities but also confirmed that in the early stages the CT could be non-specific [133]. Table 11 shows the results from several major studies of CT scanning in HSVE. Of 126 patients scanned, 88 had focal temporal abnormalities (70%). When the timing of CT scanning was given, the earliest focal CT abnormality was not evident before day 4, even though many scans were performed before this time.

Year	Patient Number	Focal Abnormality	Earliest seen (days)	Ref.
1978	9	8	6	54
1978	13	10	4	73
1980	5	5	7	133
1982	6	6	5	34
1982	11	6	7	69
1982	56	33	N.A.	278
1987	6	6	4	97
1988	18	14	N.A.	125
TOTAL	126	88	4-7	

TABLE 11: Computed tomographic brain scan findings in patients with Herpes Simplex Virus Encephalitis. Presence of a significant focal abnormality and the earliest at which a lesion was visible.

In comparative studies of EEG and CT, the EEG was invariably abnormal at an earlier stage than CT [125, 97, 34], and in some studies was more frequently focally abnormal (81% versus 59% [278]), but in most studies, similar ranges of abnormalities were found (79% versus 77% [125], 64% versus 55% [69]). When both methods have been combined diagnostically, the accuracy of focal abnormalities detected increases to 100% [69], leading to suggestions that neither should be used in isolation. However, not all cases of encephalitis with focal temporal abnormalities on CT will have HSVE [225], so findings are not pathognomonic, and 30% of patients with HSVE will have no focal abnormality.

Because of improved contrast sensitivity the imaging of limbic structures by MRI is far superior to CT, so studies have now been reported comparing the two in HSVE. Schroth et al in 1987 compared parallel MRI and CT scans in 4 cases of serological proven HSVE [223, 224]. In all cases MRI showed T₂ weighted image abnormalities much earlier than CT, in earliest on day 4 of clinical symptoms, 6 days before CT. In most patients the CT and MRI were both abnormal to some degree, but CT often showed unilateral changes, whereas MRI changes were bilateral. Although showing greater disease extent, the MRI only improved on diagnosis in 1/4 patients. A further report

highlighted the improved detection of haemorrhage, and the discrimination of abnormalities on both sides of the sylvian fissure, which are highly suggestive of HSVE [190], but no improvement in early detection rates, with isolated case reports not showing abnormalities before day 7 of the illness [190, 135].

C. SPECT Scanning

Single Photon Emission Computed Tomography (SPECT) has been described as the poor mans PET scan*. Two reports have appeared in the literature now on a total of 17 patients with HSVE. In the first report, 14 patients with acute encephalitis were studied, 6 of whom were given a diagnosis of HSVE on the basis of a 4-fold rise in CSF titres, or high static CSF titres alone [151]. All 6 had distinct areas of tracer accumulation in the affected temporal lobe, the earliest visible at day 4, which increased up to day 13, then diminished to hypoperfusion from day 37 to day 165. The 8 other non HSVE patients did not show focal hyperperfusion or progress to hypoperfusion. The second report studied 3 patients, 1 of which had a diagnosis of HSVE which was made on increased CSF titres only, and all had unilateral temporal and frontal hyperperfusion [68].

None of these cases fulfilled the criteria set down by convention for the diagnosis of HSVE, so the findings must be treated with caution. However, on the assumption that some may have been HSVE, the inference looks interesting, but the technique is not sufficiently well developed to be considered for routine use.

* PET = Positron Emission Tomography

6. Diagnosis by Brain Biopsy

Recovery of virus is the only definitive way to guarantee a diagnosis of HSVE. Brain biopsy is therefore the only direct way of making certain the diagnosis in life. As mentioned in the introduction, brain biopsy, first attempted in 1956 [63], was not successful in life until 1964 [162]. Virus presence can be inferred by cytological examination for intranuclear inclusions immunofluorescence and immunocytochemistry, electron microscopy or tissue culture [84, 40, 215, 158, 5].

For immediate results, immunofluorescence provides quick and sensitive results, with 81% sensitivity in 21 culture positive cases with only 1 false positive test [84, 5]. Immunocytochemistry became more popular because of the improved sensitivity [61, 76, 141] and reported up to 100% sensitivity [40]. The use of Cowdrey type A inclusions as a marker for HSVE has fallen from favour, since they are not specific for HSVE but are present in SSPE and other viral encephalitides [116]. Culture provides the definitive result, but takes 2-3 days [84, 5, 215].

The incidence of false negative results from biopsy is difficult to ascertain. Olson et al reported no cases of negative biopsy and positive postmortem findings, but noted that the site of biopsy was critical, in that virus was not recoverable from some sites that had gross pathological changes, and timing was also important with 1 patient having positive culture from biopsy on day 18 and negative post mortem cultures on day 24 [193].

Whitley et al in one study reported 3/79 patients with negative specimens at biopsy, positive at autopsy: these 3 had been biopsied at the "wrong site" [277]. A further 4/41 negative culture patients had other evidence of active herpes virus infections such as serology, histology, EEG and radiology. In a second study 3/109 (2%) biopsy negative patients had CSF and serum antibodies suggestive of HSVE but no patients were reported biopsy negative and post mortem positive or vice versa [274]. In Skoldenberg's

study, 47 patients underwent brain biopsy, 8 had positive antigen detection by immunofluorescence or ELISA but negative culture, and 6/25 biopsy negative patients had local synthesis of specific IgG, 5/6 having been biopsied from the frontal lobe [233].

Table 12 summarises brain biopsy reports, and shows that brain biopsy missed 16/254 patients (6.3%), although if one excludes those biopsied "at the wrong site", this drops to 3%.

Year	Total biopsy negative	Positive by other criteria*	Ref.
1967	?	4	193
1981	120	7**	277
1984	25	6	233
1986	139	3	274
TOTAL	284	16	

* - antibody titres in CSF and/or serum, histology, radiology, EEG, post-mortem

** - includes 3 positive at post-mortem

TABLE 12: False-negative biopsy results in patients with Herpes Simplex Virus Encephalitis.

The morbidity of brain biopsy is open to considerable question. Whitley et al reported an acute morbidity of 2% [274, 277] with 3 cases of cerebral oedema and 3 cases of haemorrhage in 340 cases. They did not attribute any long term neurological sequelae to biopsy. Barza et al attributed 0.5%-2% morbidity from biopsy in encephalitis [17]. Biopsy will demonstrate other diagnoses which may be missed and are treatable, but brain biopsy will be performed in up to two-thirds of patients who turn out not to have HSVE [274].

The controversy to biopsy or not to biopsy remains with each major study, followed by a plethora of correspondence [5, 36, 244, 90, 180, 275, 269, 17, 32, 101, 83, 99].

Essentially, in European centres, brain biopsy is not favoured as it is not considered an innocuous procedure and cannot be justified in patients who do not have HSVE.

Reliance is thus placed on non invasive diagnosis of high sensitivity such as evidence for local synthesis of herpes specific IgG and clinical findings. In the United States however, belief in the negligible morbidity and the absolute requirement for viral isolation to prove the diagnosis, makes brain biopsy the diagnostic tool of choice.

CHAPTER 3: TREATMENT OF HERPES SIMPLEX VIRUS ENCEPHALITIS AND ITS OUTCOME

1. Introduction

Before the development of specific anti-viral agents, there was a little effective treatment for HSVE, reflected in the paucity of information in the literature on the subject.

Surgical decompression, medical decompression using steroids, mannitol or urea and supportive therapy were all used, without much convincing evidence of any significant overall effect on outcome [215, 206], and indeed steroids may have a deleterious effect [206]. Many reports of the benefit of surgical decompression were isolated cases [63], or the surgery performed in combination with anti-viral agents, so outcome could not be ascribed to one or the other alone [207].

The introduction of specific anti-viral agents have radically altered the mortality and morbidity of HSVE, although some of the early agents did not live up to their promise. Four anti-viral agents have been used over the last 20 years in the treatment of HSVE. Only one of these, acyclovir, has stood the test of time, and remains the only agent now used.

2. Anti-viral Drugs

A. Idoxuridine

Idoxuridine (iodo-deoxyuridine, 5-iodo-2-deoxyuridine, IUDR) is a thymidine analogue, which acts by inhibiting DNA synthesis. Initially prepared for studies on cancer therapy, it was found effective in viral DNA inhibition [146]. Unfortunately it is also cytotoxic, and thus affects rapidly proliferating cells such as bone marrow, hair follicles and oral mucosa [206, 89].

Isolated case reports in HSVE appeared to confirm that efficacy, but in a review of the literature by Rappel in 1973 of 25 cases of acute necrotising encephalitis due to HSVE treated with idoxuridine, 12 (48%) died [206]. Table 13a outlines the results from the major studies reporting idoxuridine therapy on biopsy proven cases since Rappel's review of 1973.

Overall 54 patients, nearly all brain biopsy definite, were treated with idoxuridine. Mortality was 56%, with 19% suffering severe sequelae, 6% moderate sequelae and 24% mild or none. Only 24% had a reasonable outcome. Moreover, the BIH study [30], showed that not only was morbidity and mortality not much different to untreated patients, but survivors suffered severe myelosuppression, and as a result this study was terminated early.

Year	Pat. No.	Dead	Sequelae in survivors				Ref.
			Sev	Mod	Mild	None	
1970	8	4	1			3	250
1971	11	3	2	1	5		207
1972	1	1					112
1973	14	8	4		2		3
1973	4	4					194
1975	7	3	1		1	2	72
1975	8	6	2				30
1978	15*	8*	4*	2*	1*		280
1988	1	1					125
TOTAL	54	30	10	1	8	5	

* - patients were treated with either idoxuridine, ara-A or ara-C but outcome was not specified for each, so results are not included in the total

Pat. no. - patient number

Sev - severe

Mod - moderate

TABLE 13: Mortality and morbidity after treatment in patients with Herpes Simplex virus encephalitis.

a) Idoxuridine therapy.

B. Cytarabine

Cytarabine (cytosine arabinoside, Ara-C) is another pyrimidine analogue, again initially used as an anti-cancer compound, but rapidly became used as an anti-viral agent [206, 146]. Like idoxuridine, it inhibits DNA synthesis, but is also cytotoxic, and myelosuppression may be severe [89].

In Rappel's review of cytarabine in 1973 [206], of 9 cases reported, 8 died. There have been few studies since then in the use of the drug, and only 7 patients could be found treated with the drug (Table 13b), of which 1 died, 1 had a severe outcome, 2 a moderate outcome, and 3 a mild or no residual damage resulting. Overall with the high mortality (56% overall), the marrow toxicity, and the development of alternative agents, no further studies were ever performed and the use of the drug discontinued.

Year	Pat. No.	Dead	Sequelae in survivors				Ref.
			Sev	Mod	Mild	None	
1972	1			1			77
1973	9**	8	1				206
1973	2		1		1		194
1973	1				1		156
1978	15*	8*	4*	2*	1*		280
1982	2	1				1	34
1983	1			1			56
TOTAL	16	10	2	2	2	1	

** - review

* and other abbreviations - as for Table 13a

Table 13: Mortality and morbidity after treatment in patients with Herpes Simplex Virus Encephalitis.

b) Cytarabine therapy.

C. Vidarabine

Vidarabine (adenine arabinoside, 9- β -D-arabinofuranosyl adenine, Ara-A) is a purine analogue, which first came prominence when a double blind placebo trial of biopsy proven cases of HSVE in 1977 [276] showed that 30 day follow-up of 18 patients treated with vidarabine resulted in only 5 deaths (30%) versus 7 of the 10 (70%) in the placebo treated group. The morbidity in vidarabine survivors was consequently much higher than in the placebo group, and only 4 patients treated returned to normal or near normal, versus 1 in the placebo group. Mild myelosuppression was the only side effect of treatment. Apart from therapy, the only other predictor of outcome was conscious state at the time of treatment institution. Those patients in lethargy had 0% mortality, semi-coma 25% mortality and if in coma 57% mortality in the treated group.

The study was heavily criticised, because of the early termination "for ethical reasons", the short follow-up period, and the small numbers used [244, 90]. The same group was sufficiently confident of its results however, to embark on an uncontrolled trial of vidarabine alone on 75 biopsy proven cases with longer follow-up and data amalgamated from a previous trial [277]. Of 93 patients in total, by 1 year 37 had died (40%), 10 were severely damaged (11%), 16 moderately damaged (17%), and 30 were normal (32%) although normal meant only returning home with minor debilities. In this larger group, apart from conscious state at the onset of therapy, age of the patient had a significant effect on outcome, with patients over the age of 30 having significantly worse mortality and morbidity.

Thereafter, 2 major trials were undertaken comparing acyclovir and vidarabine. Before considering these, a review of the major studies using vidarabine is summarised in Table 13c.

Year	Pat. No.	Dead	Sequelae in survivors				Ref.
			Sev	Mod	Mild	None	
1978	11	5	**	**	**	**	72
1978	15*	8*	4*	2*	1*		280
1980	5	1	4				133
1981	93	37	10	16	7	23	277
1982	4	3	1				34
1982	1	1					69
1984	24	12	7	2		3	233
1986	37	20	4	8	5		274
1988	5	2	1		2		125
TOTAL	180	81	27	26	14	26	

** - 6 survivors were not ascribed outcomes and are excluded from the relevant totals

* and other abbreviations - as for Table 13a

TABLE 13: Mortality and morbidity after treatment in patients with Herpes Simplex Virus Encephalitis.

.c) Vidarabine therapy.

Of 180 cases treated with vidarabine, there was an overall mortality of 45%, with 15% severe morbidity, 14% moderate morbidity, and 22% with mild or no sequelae. So, although mortality was reduced, the number of survivors approaching normality was no better than with idoxuridine treatment, but with much fewer side effects from the drug. With vidarabine set as the standard, the next development was the introduction of acyclovir, whose value in other herpetic infections was being established.

D. Acyclovir

Acyclovir (9-(2-hydroxyethoxymethyl) guanine) was released for clinical use in 1982. It is an acyclic analogue of guanosine, discovered to have an activity against HSV in 1974. The molecular biology has been well reviewed [65] but essentially, its activation and uptake explains the selective anti-viral activity. It requires phosphorylation to the monophosphate form by a viral thymidine kinase. HSV1, 2 and varicella zoster all contain thymidine kinase, therefore acyclovir is selectively phosphorylated by infected cells. Therein it is converted to the triphosphate by guanylate kinase, and it is the triphosphate which is a competitive inhibitor of viral DNA polymerase. It does this by being incorporated into DNA, acting as a chain terminator, which cannot be excised by an endonuclease. There are little or no toxic effects, except mild reversible renal impairment in man.

Two major studies investigated the role of acyclovir in HSVE [233, 274]. Both compared acyclovir with vidarabine, since ethical reasons were put forward for not using placebo treatment. In the Swedish study [233] acyclovir 10 mg/kg/8 hourly for 10 days was compared with standard vidarabine therapy in 53 patients, 22 of which were biopsy proven. Mortality was 19% for acyclovir, with 56% of patients returning to normal life by 6 months, whereas vidarabine had 50% mortality, with only 13% returning to normal. In the NIAID collaborative study [274], 69 patients, all biopsy proven, received acyclovir 30 mg/kg/day for 10 days, versus standard vidarabine therapy. Mortality was 28% for acyclovir, with 38% returning to normal by 6 months, whereas vidarabine had 54% mortality, and only 14% returning to normal by 6 months.

Despite differences in study entry criteria, with the Swedish group accepting CSF antibody evidence as well as biopsy evidence, the mortality and morbidity figures for acyclovir and vidarabine in the 2 studies were very similar. Both studies again

demonstrated that conscious state at the onset of therapy determined prognosis - no acyclovir treated patient with a Glasgow coma scale great than 10 in the NIAID trial died. However, only the NIAID study was able to show that patients under 30 years of age had a better outlook than over 30.

Apart from these 2 trials, there are few other studies in the literature using acyclovir, presumably because the data were so conclusive. Summarising the data in Table 13d, of 74 patients treated, 24% died, 23% had severe sequelae, 8% moderate sequelae, and 45% were left with only mild disability or returned to normal life.

Year	Pat. No.	Dead	Sequelae in survivors				Ref.
			Sev	Mod	Mild	None	
1984	27	5	4	3		15	233
1986	32	9	8	3	12		274
1987	8	2	2		2	2	97
1988	7	2	3		2		125
TOTAL	74	18	17	6	16	17	

Abbreviations as for Table 13a

TABLE 13: Mortality and morbidity after treatment in patients with Herpes Simplex Virus Encephalitis.
d) Acyclovir therapy.

Acyclovir is now the drug of choice for HSVE, and should be given empirically since side effects are negligible. It has now been used safely in pregnancy in the third trimester, with no adverse effects on the foetus and a good outcome in the mother [100]. Resistance to acyclovir is now being reported, with thymidine kinase defective mutant viruses reported [51], but such mutants tend to have low virulence, and so far no resistant strains causing HSVE have been reported. The emergence of mutants has prompted some clinicians to combine acyclovir with vidarabine in the treatment of HSVE as a safety measure [24]. Another problem has been the reporting of relapse following

acyclovir therapy at conventional doses of 10mg/kg/8 hourly for 10 days in immunocompetent individuals [260], in a patient with lymphoma [216], and in a child [16], although in the last case only 8 days treatment was given. In the first case subsequent brain biopsy recovered acyclovir sensitive HSV1 and further acyclovir treatment led to recovery. These relapses, and others reviewed by Barthes [16], leads to the suggestion that treatment with acyclovir should either be for longer, perhaps 14-21 days or that any deterioration after treatment should be considered a relapse until proven otherwise.

3. Morbidity After Therapy

The comparative studies suggest that morbidity after therapy takes the same form as morbidity without therapy, but because of the improved survival, many more patients will be left with sequelae. In severe cases, patients are devastated and institutionalised. In moderate cases, the commonest sequelae appear to be epilepsy, hemiparesis and speech problems, either aphasia or dysphasia. What may be considered the most distressing problem, is the number of patients with defects of memory, personality changes and behavioural problems, which often go unrecorded because they have returned home to a "normal life". Some of the severe problems have been bizarre limbic system abnormalities, resembling the Kluver-Bucy syndrome [94], or amnesic syndromes [198, 119], but many have mild personality or behavioural changes indicative of irreversible limbic system damage, even when the encephalitis is arrested at an early stage. This makes the need for an early, quick and reliable diagnostic test of even more paramount importance.

CHAPTER 4: PATHOLOGY, AETIOLOGY AND PATHOGENESIS

1. The Herpes Simplex Virus

A. Structure

There are six herpes viruses that infect humans: HSV1, HSV2, varicella, cytomegalovirus, Epstein-Bar virus and human herpes virus 6. Herpes viruses are defined on the basis of the design of their virions. All have a core consisting of double stranded DNA, and DNA binding proteins, surrounded by a capsid of 162 capsomeres in the form of an icosahedron. Around this is an amorphous mass of proteins called the tegument, whose properties and functions are unknown, and enclosing all is the envelope, which is acquired as the DNA containing capsid buds through the inner nuclear membrane of the host cell. This envelope contains only virally derived proteins [197].

Herpes viruses exhibit great differences in the size of their DNA and base ratios. HSV1 and 2 contain linear DNA, with a molecular weight of approximately 100×10^6 Daltons coiled into a doughnut shape. There is about 50% homology between the HSV1 and 2 genomes, and sufficient variability of nucleotide sequences between strains that restriction enzymes can be used to differentiate isolates from separate individuals. The envelope of the virus contains embedded within it at least 5 glycoproteins which mediate attachment and penetration into the cell. Glycoprotein B is required for infectivity, glycoprotein C binds to C3b component of complement, glycoprotein D is the most potent inducer of neutralising antibodies, glycoprotein E binds the Fc fraction of IgG, and glycoprotein G whose function is unknown is used for type specificity between HSV1 and 2[197].

B. Genetics

The HSV genome is 152 kilobase pairs in size, and consists of 2 components, the L and S sequences containing unique sequences, flanked by inverted repeats [197]. The 2 components can invert relative to each other, thus creating 4 isomers, present in equimolar proportions in infected cells. The function of the isomerisation is unknown, but does not affect infectivity or latency. The genome codes for a number of proteins and about 70 are expressed during productive infection, and are grouped on the basis of a highly regulated cascade of expression. The α genes code for proteins and are expressed earliest in infections. They are induced by one of the tegument proteins (α trans inducing factor or α TIF). The function of most of the proteins is unknown, but the $\alpha 4$ is required for the expression of subsequent protein groups. The β genes are expressed after 5-7 hr., require prior synthesis of α proteins, are mainly involved in nucleic acid metabolism, and code for the regulatory proteins and enzymes, including the alkaline nuclease. The γ genes code for the structural proteins, some of which are completely dependent on viral DNA being synthesised, like glycoproteins C and E, and others are expressed irrespective of the presence of replicating viral DNA, such as glycoproteins B and D, α TIF and capsid proteins. Once the cascade is set off, autoregulation probably plays a role in determining how much viral synthesis and cell destruction takes place.

C. Infectivity

The virus attaches itself to the cell via the glycoprotein spikes. It is likely that cell receptors exist, but are difficult to identify. In neurones, the receptors are located on the synaptosome [258, 259]. Once attached, fusion occurs, and virion components move through the cytoplasm. The capsid moves to nuclear pores, and virus DNA is ejected into the nucleus. Circularisation of viral DNA occurs immediately, then the transcription cascade commences, beginning with the α genes. α mRNA's are transported to cytoplasm and translated, the resulting proteins initiating β genes transcription. Once β genes are transcribed and α proteins synthesised, viral DNA replications occurs via a rolling circle mechanism. The assembly of viral capsid occurs in the γ phase, the capsids are filled with viral DNA and proteins, the tegument attaches and then buds through the nuclear membrane acquiring the glycoprotein envelope. As the virion moves through the cytoplasm using the endoplasmic reticulum and golgi apparatus, glycoprotein processing occurs and the final complete virus leaves the cell. The cell surface also contains viral glycoproteins which can be recognised in immune cytolytic reactions [47].

2. Latency

A. Latency in Humans

Even before the causative agent of herpes simplex cutaneous infections was known, the association of recurrences with peripheral nerve trauma was observed by Cushing in 1905 as a result of the use of nerve section to treat trigeminal neuralgia [52]. In between recurrences, biopsy of tissue consistently failed to yield any virus, thus leading to the conclusion that virus may remain in a latent state in the ganglia after the local lesion had healed [212]. Latency is defined as the need to induce a virus to multiply, without it being recoverable in infectious form. Although animal studies suggested that the trigeminal ganglion was capable of harbouring herpes simplex virus, isolation from human cadavers was not shown until 1972, with the cocultivation of explants from the trigeminal ganglion from 2 of 23 autopsied patients, one of whom had no evidence of herpetic infection at the time [18]. No virus was recovered from the choroid plexus in any of the patients studied. The relatively poor recovery was likely due to the cocultivation technique, as in 1973, using direct culturing of ganglion cells, 6 of 7 ganglia from autopsied patients produced HSV. Two of the patients had a history of either trigeminal neuralgia or oral herpes, but none had evidence of herpes at the time of death [13].

Latency in trigeminal ganglia has been confirmed by other workers using both culture [211, 267, 11] and detection of viral RNA [50, 71]. Other sensory and autonomic ganglia have also successfully yielded cultures of HSV, including sacral [11], superior cervical and vagus [267], and autonomic [212] - see table 14. The yield from the trigeminal ganglia however, is by far the highest at up to 80%. Careful studies show the neurones to be the source of the virus, but they do not display any known virally coded polypeptides and infectious virus cannot be recovered directly from the ganglia. Herpes virus has since been detected in other neural tissues, including brain smears from elderly

patients with psychiatric illnesses using DNA probes [228], and in temporal lobe smears from immunosuppressed patients with a history of HSV infection, but not in non-immunosuppressed patients or immunosuppressed patients without a history of herpetic infections [219].

Site of detection	Method of detection		Ref.
	Culture	DNA-probe	
Trigeminal ganglia	+	+	18
Sacral ganglia	+		11
Cervical ganglia	+		267
Vagal ganglia	+		267
Autonomic ganglia	+		212
Whole brain smears		+	228
Temporal lobe smears*		+	219
Hippocampus**			76

* - Immunosuppressed patients only

** - Immunohistochemical detection

TABLE 14: Sites of detection for latent human herpes simplex virus.

These findings suggest that viral latency may be more widespread in neural tissue, or that in the cases of extra ganglionic detection, they represent reactivation secondary to immunosuppression and aging. The finding of HSV antigen in the granule cell layer of the hippocampus in one case of Alzheimer's disease opens up further the debate on how widespread herpes virus may be, latent or otherwise [76]. So far other immunohistochemical studies on temporal lobes for HSV antigens have failed to find evidence of herpes in Alzheimer's patients, but this does not preclude latency [209].

B. Latency in Animal Models

Proof that the trigeminal ganglion could harbour latent virus came initially from studies in mice [238] and it was the development of the mouse model system that has led to most of the understanding of latency and the definitions that apply to the concept. Recovery of virus by cocultivation and explantation from experimental animals has shown that both sensory and autonomic ganglia can support latent virus infection [200, 139], but in addition in-situ hybridisation with DNA probes has shown virus in the central nervous system in up to 50% of mice with latently infected trigeminal ganglia [35, 205], and infectious virus can be recovered from brain following massive immunosuppression [120]. Subsequent studies have also shown that mRNA indicative of some transcription can be detected in latently infected trigeminal ganglia [247, 243], and that only one particular mRNA transcript is found in latency, the infected cell protein O (ICP-O) transcript [243].

Establishment of latent infection using inoculation takes place via 3 methods, peripheral, intracerebral or systemic. By far the most productive route is peripheral, using the eye [212], but the ear, nose, lip, footpad and vagina have all been successfully used. Initially viral replication occurs at the inoculation site, but within a few days the respective ganglia may show infective virus. In surviving animals, once virus disappears from the peripheral site, the ganglia no longer contain infectious virus, and only techniques for reactivation show viral presence. It was these observations that led to the working definition of latency described at the beginning of section A. With herpes simplex viruses, it would appear that only neurons can harbour latent virus with no other tissue so far being shown to do so.

C. Genetics and Mechanisms of Latency and reactivation

How latency is achieved, what maintains it and what switches on viral replication has not yet been fully explained. Less than 1% of the neurones in a ganglion harbour virus in the latent form [212], and what determines what neurones are infected is unknown. There is some evidence to suggest that host factors may determine susceptibility to latency. Mice and rabbits infected via identical routes and identical HSV1 strains produce differing proportions of neurones infected as well as differing levels of latency. Virus DNA present in latent neurones is in a different form to that present in actively infected cells. The DNA is non-linear, either circular or concatameric (i.e. end to end) and each cell may contain hundreds of copies. Whether the DNA is integrated or episomal is still not clear [91]. Studies on viral mutants, such as temperature sensitive, thymidine kinase deficient and 'dispensable gene' mutants have not yielded any specific feature that is essential for viral latency [212]. Studies using complementary DNA probes have identified latency associated transcripts. An ICP0 probe was the only one to react with mRNA during latent infection, and the sequences expressed were 'anti-sense', that is, on the opposite strand of the ICP0 encoding gene [50, 239]. Latent HSV2 has been shown to behave in an identical fashion with the same transcripts being evident [243, 247]. ICP0 is an immediate early α gene, thus restriction of transcription of the genome to this region may be the mechanism of maintenance of latency, by preventing initiation of the normal cascade.

Numerous insults cause reactivation of latent infection, including surgery, emotional stress, febrile illnesses, x-rays, ultraviolet light and minor trauma [113, 48, 186]. Even asymptomatic patients can shed HSV into the oropharynx, so one must be careful in using the criteria of viral isolation from non neurological sites as evidence of causation for neurological illness, and more importantly, coincidental reactivation of ganglionic and possible cerebral or brain stem lesions may accompany other pathology. The likelihood of reactivation may be viral dependent and neurone dependent. Studies of

recurrence rates of HSV1 and 2 affecting both trigeminal and sacral ganglia, showed that sacral ganglia were more likely to harbour reactivating virus than trigeminal, and HSV2 infections more likely to recur than HSV1 [144].

3. Pathogenesis of Herpes Simplex Virus Encephalitis

A. Evidence from Human Studies

For many years HSVE was thought to be a primary infection because of the assumption in early insensitive serological studies that only primary infections were associated with a rise in serum titre of antibodies [154]. It is now known that encephalitis may result from a primary infection, reinfection or reactivation of latent infection [271]. Restriction endonuclease analysis of HSV DNA isolated from the brain and orolabial sites in 8 patients from this study, showed paired isolates were identical in 5 but different in 3 others. One patient had unequivocal evidence of primary infection, with identical isolates, 5 had a history of recurrent labial herpes, 3 with identical isolates, and 2 had no history but a serological response, 1 with identical isolates. In addition there were no obvious similarities between isolates from different patients to suggest a common denominator for neurovirulence. The association of encephalitis with reactivated latent infection was first suggested in a review of 18 patients with HSVE, 3 of which had recurrent herpes labialis [154]. Since then, other studies have confirmed the association, but since 90% of adults have serological evidence of infection in the past, and up to 80% of autopsied cases have latent herpes virus in the trigeminal ganglion, and up to 47% of patients give a history of recurrent cold sores, a history of labial herpes in encephalitis given the prevalence in the community is not surprising [227].

The route of viral entry into the brain is still speculative in humans, and table 15 illustrates various routes proposed [114, 115, 91]. Pathological studies have demonstrated that limbic structures are preferably infected, and that fronto orbital, medial and inferior temporal lobes are severely damaged. This observation led Hurst in 1936 to postulate that the virus gained entry via the olfactory nerves [106], but little objective evidence was available from human encephalitis studies to confirm this [114]. Several pathological studies since then, have shown that not only herpes virus

recoverable from the olfactory mucosa and bulbs [84, 60, 254], but also olfactory tracts [61], with confluent involvement of limbic structures [76]. This is not a universal finding however, and it has been postulated that the involvement of the olfactory system may be secondary [76] although proposals of brain stem projection to olfactory routes as the explanation are unconvincing [53].

Origin	Pathway	Destination
Olfactory bulb	Olfactory tract	Limbic system
Trigeminal ganglion	Tentorial branches Trigeminal nerve	Middle fossa
Trigeminal ganglion	Brainstem nucleus	Limbic system
Brain tissue/ Hippocampus	----->	Limbic system

TABLE 15: Possible routes of spread of herpes simplex virus into the human central nervous system.

An alternative or complementary route has been postulated on the basis of latency of HSV in the trigeminal ganglion, and the observation of selective frontal and temporal lobe involvements. Penfield and McNaughton in 1940 showed that the dura in the middle and anterior fossa is innervated by the tentorial branches of the trigeminal nerve [196]. It has thus been postulated that latent virus is reactivated and travels along these branches to the dura in the appropriate fossa, and thence, 'comes into contact' with the brain [55, 113]. This does not satisfactorily explain the strict limbic anatomical boundaries that HSVE respects [76], and why other areas equally well supplied by the trigeminal nerve divisions in the middle and anterior fossa are spared. An alternative hypothesis proposes that the localisation is due to spread from the trigeminal ganglia to the brain stem nuclei, from whence virus connects trans-neuronally to limbic structures by well documented pathways [53]. Those pathways are specifically mono-amine containing, and it may be that these neuronal types are particularly susceptible to viral

infection and spread, at least in animals, if not humans [189]. Evidence for brain stem involvement in humans is recorded, but does not appear to be that common, and in one large immunohistological study of 29 cases of HSVE, there was no evidence of any viral antigens in the meninges, trigeminal ganglia or route entry zones of the trigeminal nerve [76].

As described earlier, latent DNA has been isolated from brain smears and viral antigen found in autopsies of 'normal control brains' so it is possible that infection could result from reactivation from these sites. As yet then, there is no proven mechanism for HSVE, but as one reviewer has commented, it is reassuring to know that since most of us harbour a potentially fatal virus in the trigeminal ganglion, the occurrence of oral herpes means at least the reactivation has not gone in the reverse direction [55].

B. Evidence from Animal Models

It was Goodpasture and Teague in 1923 who first demonstrated that HSV could be transmitted along peripheral nerves to the CNS in a rabbit model [93]. By corneal and masseter inoculation, localised brain stem lesions were produced in appropriate sensory and motor areas, and further experiments suggested that the 'axis-cylinders' provided the route, with viral propagation within the axon causing spread [92]. This model successfully showed that the trigeminal nerve branches acted as a portal of entry to the trigeminal ganglion and thence trigeminal root entry zone and connections [12], but the actual means of transfer of virus from periphery to CNS remained speculative, with the axon itself, perineural and endoneural cells lymphatics and perineural spaces all being proposed [114]. Baringer and Griffiths showed that it was the transport centripetally along the 'axis-cylinders' that was the primary event in a corneal inoculation model in the rabbit, and that schwann cell and satellite cell infection was secondary. Virus then spread to neurones and astrocytes in the associated tracts [12]. This primary axonal transport mechanism was confirmed in mice [136]. Within the central nervous system, spread of virus has been shown to take place intra-axonally in a retrograde fashion [67, 142], and in a rat model adjacent neuronal structures which were not axonally connected were unaffected [8].

Many other animal models have now appeared in addition to examine the pathogenesis of HSV invasion of the CNS and are summarised in Table 16. All show that the transfer of virus takes place along anatomically connected pathways from 1st to 2nd and 3rd order neurones, using a variety of portals of entry resulting in a variety of pathological models.

Animal	Route of inoculation	Sites of pathology	Ref.
Rabbit	Cornea	Brain stem	93
Rabbit	Masseter	Brain stem	93
Rabbit*	Olfactory	Temporal lobes Hippocampus Brainstem	221
Mouse	Snout	Trigeminal pathways Basal ganglia	137
Mouse	Olfactory	Olfactory bulb Trigeminal pathways	75
Mouse	Hypoglossal	Reticular formation Inferior olive Cerebellum	255
Mouse*	Hippocampus	Limbic system Olfactory route	171
Rat	Intraocular	Optic radiation Hypothalamus Trigeminal pathways Oculomotor nuclei	43

* - pathology similar to human distribution

TABLE 16: Animal models of pathogenesis of spread of herpes simplex virus to the central nervous system.

None of the peripheral inoculation models however, have produced pathology which in any way resembles that seen in the human. This lack of appropriate pathology has prompted some groups to try more invasive methods of infection in order to create a more representative model. Thus intra-ocular inoculation [43] and direct injection into the hippocampus [171, 169] result in extensive encephalitic changes, which, especially in the latter model, affect predominantly the limbic structures, with notably involvement of the olfactory nucleus in some cases, indicating that olfactory bulb involvement could be a secondary phenomenon. There has only been one model developed, however, that reproduces a 'physiological route' of viral invasion with pathology that more closely reproduces human limbic system involvement. This model involves direct inoculation

into the olfactory bulb in rabbits [221] and resulted in a predominantly limbic involvement with temporal lobe seizures, and although brain stem pathology was present, it was rarely severe or showed much in the way of destruction. Thus, animal models are getting close to supplying a model of human disease in terms of pathology, but there are other factors involved in determining how and why neurones get infected, and determining the differing patterns of pathology.

The neurotropism of HSV has now been shown to result from specific viral receptors located probably on synaptosomes of neurones and glia [258, 259], and not by pinocytotic vesicles as was suggested earlier [138]. In fact the virus has a greater affinity for glial cells than neuronal bodies, perhaps secondary to receptor density, and thus may explain the ready uptake of virus from glia from neurones, but not in the reverse fashion [255]. Entry into the sensory or motor nerve ending in the primary infection is thus relatively easy, given a sufficient inoculum, and cell to cell transfer along synaptic routes explain the pathological distribution. This may also explain the abrupt border seen at the trigeminal root entry zone, where peripheral nervous system and central nervous system abut.

Table 17 summarises those factors which are shown to influence the neurotropism and pathogenesis of HSV viral infections in animal models. Differing cell types have different susceptibilities to infection. Although the peripheral Schwann cell in the trigeminal model contains virus, there is rarely destruction, whereas neurones and astrocytes on the CNS side of the trigeminal root are readily infected and frequently excite a destructive demyelinating response [253, 102, 157]. Despite human pathological reports, in animal studies oligodendrocytes show only rarely viral infection [253] but where they do, there appears to be differential susceptibility of oligodendrocyte populations to HSV infections [121, 122]. The peripheral nerve also contains fibres with differential sensitivities to HSV [157].

Level of action	Factors influencing outcome	Possible mechanisms	Ref.
Cellular	Neurone vs neurone	Receptor density	170
	Neurone vs glia	Receptor density	255
	Glia vs glia	?	121
	Peripheral vs central	?	157
	Pathway	Anatomical Neurotransmitter	8 189
Viral	Neurovirulence	Genetic	221
Host	Intraspecies	Genetic	160
	Interspecies	Genetic	241
	Age	Receptor density	173
		Axoplasmic flow	173
		Anatomical maturity	173

TABLE 17 : Factors influencing the neurotropism and pathogenesis of herpes simplex virus infections in animal models.

Different neuronal cell populations have selective vulnerabilities to HSV infection with hippocampal injection resulting in widespread infection of some projections and not others [171] and cerebellar injection resulting in little or no pathological changes [169]. Large neurones appear to take up viral antigen more avidly than small neurones [8]. The basis for neuronal preference may lie in the density or distribution of receptors [170] or in particular, neurotransmitter related structures, with monoaminergic neurones preferentially involved [137, 189].

The virus itself displays strains of differing neurovirulence, probably related to distinct genetic loci as demonstrated by the elegant work of Stevens and co-workers [283, 286, 287], with some reports linking also the regions coding for glycoprotein B and DNA polymerase [91, 221, 43]. Seizure production is related to the use of highly virulent strains. Host-genetic factors also determine the level of resistance to infection, with at least 3 genes responsible in mice, and the effect probably mediated through immune mechanisms [160].

Knowledge of the role of HSV in demyelination associated with the encephalitis has stemmed from animal model work. Following trigeminal inoculation in mice, demyelinated areas appear within the trigeminal pathways relatively late and persist for months [139] with peripheral myelin preserved. Different strains of mice have different susceptibilities to demyelination with resistant strains having very focal areas and susceptible strains widespread multifocal involvement [121, 122]. The absence of recoverable virus at the time of demyelination suggests an immune mediated mechanism. Antibodies to myelin basic protein (MBP), and delayed type hypersensitivity reactions to HSV and MBP are found in mice models to induce demyelination, supporting the concept of immune mediated responses. Studies of the immune cell infiltrates into the brain and perivascular area in mouse models shows that although oligodendrocytes, astrocytes and microglial cells are infected, the demyelination present by far outweighs the oligodendrocyte involvement, and the perivascular infiltrates of T-cells and macrophages were close to areas of massive myelin loss, suggesting immune mediated myelin damage

accounted for as much of the pathology as direct viral cytolysis and inhibition of cellular function. Re-myelination can take place, at least in the spinal cord, and appears to be mediated by both Schwann cells and oligodendrocytes to such an extent that complete recovery can be possible [237].

C. Animal Models for Diagnosis and Therapy

Since animal models are proving successful in at least partially reproducing human pathogenesis and pathology, attempts are being made to extrapolate results for diagnostic purposes. Radiolabelling of anti-viral compounds to 'locate' virally infected cells is possible [218], and with SPECT scanning offers a promising avenue of research now that reasonable models are available [43]. In preliminary work, a rat model has been successful in radiolabelling infected areas of brain and the radioactivity detected with a gamma camera [42]. Cell culture work also suggests that acyclovir therapy would not interfere with radiolabelled anti-viral drug uptake in the early stages, thus the use of such diagnostic tools would not compromise therapy [252].

A resurgence in interest in using CSF cells has been prompted by the detection of viral specific antigen in CSF cells using immunofluorescence in mice [27]. Seventy-five percent sensitivity was reported, but specificity not tested, and observers may disagree on border line cases. However, it must be stressed that it is yet to be proven that this animal model can successfully translate into human cases given the outcome of previous work with CSF cells.

4. Pathology of Herpes Simplex Encephalitis

Pathological studies, both macroscopic and microscopic, of the changes in adult HSVE are numerous and only a brief summary is warranted. Since only severe cases come to post mortem, findings usually reflect the severe devastation. Macroscopically, the brain is often swollen [21] with softness and mushiness of parts of the cortex [103], and sometimes the entire brain is very soft [236]. There are haemorrhagic necrotic areas, often cavitated, most frequent in the medial temporal lobes and fronto-orbital area and less so occipital and parietal areas [177], with changes being unilateral or more frequently bilateral. The cingulate gyri and insula are invariably involved, the thalamus and pons regularly involved, and the brain stem and cerebellum infrequently and sometimes never affected [177]. The hippocampus and basal ganglia, if not macroscopically involved, are invariably microscopically involved [4]. In sections, multiple haemorrhagic foci with marked necrosis are present, especially in the cortex and adjacent white matter. These changes can suddenly demarcate to relatively normal tissue, although in general there is some gradation. Overlying involved areas is a severe leptomeningitis, with a mainly lymphocytic macrophage infiltrate. The microscopic changes parallel macroscopic, but are often more extensive. Histologically, changes are diffuse with selective necrosis of grey matter and spread into white matter adjacent to most severely affected areas. The marked necrotic changes present could, like the macroscopic view, suddenly demarcate microscopically to relatively normal tissue with just scant inflammatory border changes. Changes are often perivascular with lymphocytic cuffing, and neuronophagia is common with sometimes whole areas replaced by phagocytes and plasma cells [103]. Microscopic involvement of the brain stem, though recognised, is often disputed as being primary, and attributed to secondary tentorial herniation [4]. Intranuclear inclusions (Cowdry Type A) are commonly found throughout the histologically involved areas, mainly in oligodendroglia, less commonly in ganglia cells neurones and astrocytes [103, 177, 66].

The first study to look systematically for this distribution of viral antigen in brain, was that of Esiri in 1982 [76], although 2 studies prior to this had demonstrated viral antigen in brain, these had been limited to temporal and frontal lobes [141, 20]. In the study of Esiri, the unilateral origin of the disease was confirmed, and the limbic system shown to be primarily infected with viral antigen staining, although occasionally other structures contained viral antigen such as the post central cortex. The asymmetry of involvement was preserved, even though most changes were bilateral. Those areas showing the most severe destruction and inflammation were not necessarily those containing viral antigen. By the 3rd week of illness, viral antigen could no-longer be detected. Viral antigen was found in all types of glial cells as well and neurones.

In terms of disease progression, the disease appeared to start in one temporal lobe, hippocampus, amygdaloid nucleus complex, and cortex close to the entry of the lateral olfactory stria, insula and cingulate gyrus. No clear indication of the initial structures infected could be definitely found, although the olfactory pathway was frequently involved early. There was never any evidence of viral antigen in the trigeminal pathway, so this study tended to support the concept of olfactory entry rather than trigeminal. One area of brain severely involved in patients and in one control brain also, was the granular cell layer of the dentate fascia in the hippocampus. The possibility was raised that this could be an area of latency.

Later studies, using more sensitive methods, have confirmed these findings [40], with immunostaining in all glial and neuronal cell types, but none in perivascular and leptomeningeal lymphocytes. The macroscopic distribution was the same, with sharp demarcation away from limbic system structures and patchiness of antigen presence. Viral antigen has however, been confirmed in the brain stem in two pathological studies, one exclusively in the spinal trigeminal tract and associated nuclei with no antigen detection above or below, suggesting trigeminal route of entry [143], and one confined to

nuclei and tracts associated with the glossopharyngeal and vagus nerves, with no involvement above and below [213]. Only one other study has reported brain stem pathology as part of the more typical limbic picture [58].

Thus, both macroscopically, microscopically and immunohistochemically, the limbic system is the first and predominantly involved area, initially unilaterally, but very quickly bilaterally. The route of entry is difficult to ascertain, with olfactory being either primarily or secondarily involved, and despite brain stem microscopic changes, viral antigen is only occasionally reported as found. Viral antigen can disappear from undoubtedly affected sites, which may explain the disagreements between some pathological studies. Antigen is found in anatomically and functionally contiguous areas, suggesting cell to cell transfer. The disappearance of antigen, and also the patchy nature of involvement of the disease process, could explain the negative biopsy results seen and perhaps the difficulty in recovering virus from the CSF.

One final point sometimes raised is the question of demyelination, with some authors reporting patchy demyelination in white matter [207], while most fail to report this or report that what is seen is secondary disintegration in perivascular areas occupied by mononuclear cells [103]. One report of a case of HSV brain stem encephalitis, found a secondary acute haemorrhagic leucoencephalitis distant from the site of infection and postulated immune mechanisms [143]. A further report documented a post-viral encephalomyelitis following successful treatment of HSVE with vidarabine [130]. HSV antigens have been sought in demyelinated areas in multiple sclerosis without success [163] so demyelination associated with HSV appears to be very rare in humans.

CHAPTER 5: CHEMILUMINESCENCE

1. Introduction

A. Definition of Luminescence

Luminescence is the chemical production of light. It is the only reaction where chemical energy is converted into light quanta, the decay of the molecule from an excited state back to ground state being accompanied by light emission. Two main forms of interest exist: bioluminescence, which is the form found in biological systems, where catalytic proteins increase the efficiency of the system to above 90% in some cases, and chemiluminescence, the purely "chemical form", whose efficiency is under 1% for many reactions [281], where efficiency is defined as the actual yield of photons versus the theoretical yield.

Luminescence must be distinguished from fluorescence and phosphorescence. In the former, irradiation of the substance with the light of a given wave length results in a promotion of the molecule to an excited singlet state and the release of radiation of a longer wave length (with lower energy). No chemical reaction takes place. In phosphorescence, which is a special form of fluorescence, light emission is long lived, since the energy state reached is a triplet state and thus different to that achieved in fluorescence [281].

B. History

References to luminescence are dotted throughout ancient texts, with the Chinese describing fire-flies (glow-worms) around 1500 BC, and Aristotle describing the bioluminescence of dead fish and fungi in *De Anima* in the 4th century BC. Observation gave way to experimentation in Robert Boyle's major work on bioluminescence published in 1667 by the Royal Society, which showed that oxygen was an essential factor for luminescence. A century ago, luciferin and luciferase were described by Dubois, who later lit a room at the Paris exposition in 1901 with bioluminescence.

Chemiluminescence has a relatively short history. Lophine and pyrogallol were not described until 1877 and 1887 respectively, and luminol and lucigenin not until 1928 and 1935 respectively [270]. Despite this, luminescence has gained wider acceptance than bioluminescence for reasons given in the next section.

C. Bioluminescence

The original role of bioluminescence has been suggested to be a detoxification mechanism to eliminate oxygen radicals [281]. This suggestion appears to be confirmed by the fact that bioluminescence is confined to lower organisms, and is absent in reptiles, bird, marsupials and mammals, as well as the more evolved members of the plant kingdom (see Table 18).

Families with Luminescence	Families without Luminescent members
Unicellular organisms Bacteria Fungi Sponges Jellyfish Corals Worms Squids Snails Deep-sea fish Centipedes & Millipedes	Reptiles Birds Marsupials Mammals

TABLE 18 : Luminescent organisms. (From Wood²⁸¹)

Although the firefly and glow-worm are the best known luminescent organisms, the majority are sea-living organisms, ranging from bacteria and plankton to fish. Two thirds of the organisms in the upper 2,000 metres of oceanic waters are bioluminescent, but fewer than 1% have been studied. The enzymes involved are all named luciferases, and the substrates luciferins. The colour of light emitted is dependent on the luciferase; spontaneous decay of luciferins always produces blue light. Five different bioluminescence chemical systems exist [270]. The pyridine-nucleotide link system (e.g. marine bacteria); adenine-nucleotide link systems (e.g. the fire-fly); enzyme-substrate systems (e.g. the clam); peroxidase systems; and "pre-charge" systems (e.g. jellyfish). The two most widely applied to analytical chemistry are the

adenine-nucleotide and pyridine nucleotide based systems. These have been extensively reviewed [14, 270, 281], and since they are not of relevance to this study no more time will be devoted to these, except to explain the limitations of their usage.

Although applied to the analysis of many substances, both substrates and enzymes, use of bioluminescence in vitro is limited by the sensitivity of the systems to anti-microbial agents in laboratory reagents (such as merthiolate and sodium azide). In addition, reagents are often expensive and label synthesis complicated. This limits the storage and stability of bioluminescent reagents sufficiently for the much less energy efficient system of chemiluminescence to be the method of choice.

D. Chemiluminescence

Many organic compounds are chemiluminescent upon oxidation, and it is probably a universal property, albeit very weak for most [270]. Chemiluminescence can be based on gas, liquid or solid phases, but liquid phase is the most useful. Several systems are used in analytical work.

1. Cyclic Hydrazides.

Luminol, a cyclic diacylhydrazide, was the first described, and is the only type to luminesce. Most derivatives, such as isoluminol, have reduced efficiency (10% of luminol) but occasionally some, like annelated analogues, are more efficient.

2. Aminonaphthyl hydrazides.

These have not found great usage analytically.

3. Acridinium derivatives.

These have the attraction of requiring only hydrogen peroxide for activation and thus are simple and cheap, but the consequence of this is a less stable compound. Their quantum efficiency is low at 1%-2%. They can however, be catalysed by some metal ions ineffective on luminol, and some acridinium phenyl carboxylates can be tailored to suit different pH environments.

4. Diaryl oxalates.

These have high quantum efficiencies, often above 30%, but the compounds themselves are not chemiluminescent, and an additional fluorescent molecule is needed for energy to be transferred to. Their light output lasts several hours, so they have been used for cold light sticks in camping and caving.

The advantages of chemiluminescent reagents is their stability over long periods of time, even in the presence of anti-microbial agents. They are cheap, and because the molecules are small, do not interfere unduly with the structure of antigens or antibodies. Full control of light emission is possible, unlike radioactivity. However, their quantum efficiency is low, at only 1%-2% for some acridinium salts, up to 50% for some acridinium esters. The measurements cannot be repeated, and quenching occurs with most, although acridinium derivatives can have this minimised.

Overall the benefits outweigh the drawbacks, so chemiluminescent assays are more widespread in use. Because the chemistry has been extensively studied, the luminol and isoluminol systems were first choice reagents and will be considered in more detail.

2. Luminol and Isoluminol based chemiluminescence

A. Labelling

Both luminol and isoluminol may be bound to proteins or haptens by a variety of methods [282, 87, 270]. Diazotisation of luminol and isoluminol have been advocated as an effective means of labelling IgG, with no resulting loss of luminescence when bound to the antibody [270]. However, several reports show that such labelling, and especially the pH required to label, results in greatly reduced binding capacities of the antibodies [87, 282]. Bi-functional coupling agents, such as gluteraldehyde can be effective, or with luminol the conversion of the aromatic amino group into an isothiocyanate group. Isoluminol derivatives appear useful for labelling, particularly 6-(N-(4-aminobutyl-N-ethyl) 2,3-dihydrophthalazine 1,4 dione) hemisuccinamide (ABEI-H), and 7-N-(4-aminobutyl-N-ethyl) naphthalene 1,2 dicarboxylic acid hydrazide (ABEN), the hemisuccinamide form being used (ABEN-H). Esterification of the label is the best method of coupling, via N-hydroxysuccinamide and dicyclohexyl carbodiimide.

When used as labels, they are stable, not affected by azide or detergents, and can be bound to a variety of haptens and proteins, including IgG, without apparently affecting biological activity or reducing luminescence. Indeed in some systems, luminescence has been increased, especially with isoluminol-biotin conjugate when bound to avidin. In some however, such as binding to thyroxine, light output may be decreased up to 50-fold.

One disadvantage in using the chemiluminescent molecule as the probe, is that its concentration will always be limited, therefore the light output is short-lived, producing a flash of light. An alternative to this is to use an horseradish peroxidase as the label, and using this to catalyse the reaction of the luminescent substrate which is in excess. This method can be applied to luminol, and has potential light output 10 times that of isoluminol.

B. The Chemical Reaction

Light production in chemiluminescence is dependent on an oxidation step, and each luminogen has its own optimal oxidation system. Luminol and isoluminol and derivative can use a variety of catalysts, such as Cu^{2+} and $\text{Fe}(\text{CN})_6^{3-}$, but the most efficient is haem. Haem catalysts include peroxidases, such as horseradish peroxidase, cytochrome C and a derivative of cytochrome C known as microperoxidase MP11 (both pseudoperoxidases), hematoporphyrin IX, and finally hemin. Horseradish peroxidase and hemin are relatively cheap, even if less efficient than some of the others.

The pH of the system is fairly critical, but varies from system to system. For most, pH 11 is the optimum, and is best supplied using sodium hydroxide, sodium tetraborate or sodium triphosphate. The higher the pH, the higher the light output and the slower the kinetics. A supplier of nascent oxygen is the final requirement: hydrogen peroxide is the commonest used, but perborates and persulphates have been successful.

The chemical reaction proceeds as detailed in Figure 3. Because the reaction is instantaneous, the addition of oxygen or the O^\cdot radical usually takes place in the measurement device. For each system, reagents, pH, timing, proportions have to be optimised, as there is no predictable response if one varies any of the components.

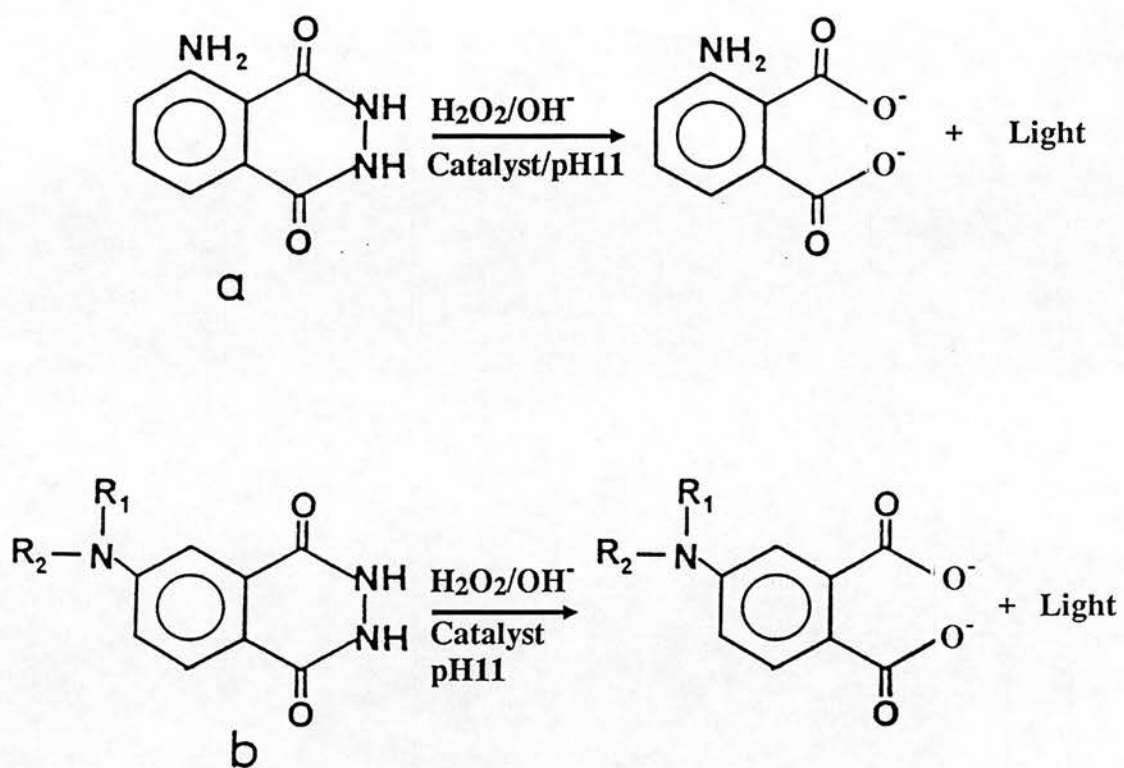


FIGURE 3 : Chemical reaction of luminol and isoluminol chemiluminescence

a) luminol

b) isoluminol - $R_1 = \text{H}$

- $R_2 = \text{H}$ - isoluminol

C. Assay Systems

Many assay systems exist, based on both solid and liquid phases. The disadvantage of liquid phase systems, is that prior extraction of the substance of interest is usually required, as many biological samples contain materials that quench light output or have nascent oxidisers, such as haemoglobin. The terminology used to describe assays is also not yet uniformly applied, but is similar to that used in radioimmunoassay.

1. Chemiluminescent immunoassay (CELIA).

Labelled antigens are used in competition with test antigen for specific antibody. This can be either solid or liquid phase, but prior extraction is required. This has been applied to steroids and their derivatives in plasma and urine. [284]

2. Immunoluminometric assay (ILMA).

This solid phase sandwich assay has a labelled first antibody, and has been used for α foeto-protein, and β 1 pregnancy specific glycoprotein.[288, 289]

3. Immunoluminometric labelled second antibody (ILSA).

As for ILMA, but a labelled second antibody is used for further amplification, and where the first antibody cannot be labelled, such as when loss of sensitivity occurs. This has been applied in addition to thyrotropin analysis. [289]

4. Solid phase antigen luminescence techniques (SPALT).

In this antigens, or particularly haptens with only one antigenic determinant in non extractable samples are coupled to the solid phase. The first antibody is then detected by a labelled second antibody.

5. Chemiluminescence enzyme immunoassay (CELISA).

Peroxidase conjugated antibodies are used to catalyse luminescence in the presence or absence of enhancing agents to prolong light output. These have been extensively used in competitive assays and sandwich assays and have been adapted for use in the detection of herpes virus antigen in biological materials. [202, 203].

Other assay systems do exist, using cofactors, such as the measurement of biotin, or enzyme multiplied immunoassays, for drug measurements, but are not of relevance to work described here.

D. Sensitivity of Luminol and Isoluminol Chemiluminescence

Assays using chemiluminescence were introduced as alternatives to radioimmunoassays, to help dispense with the need for radioactive sources and handling of radioactive materials. To gain acceptance, they have had to be of similar, if not greater sensitivity, and this is now becoming the case. For example, commercial RIA's for TSH have a sensitivity of 0.3 mU/litre with a working range of 1-50 mU/litre and at best a sensitivity of 0.07 mU/litre [268]. Monoclonal based immunoradiometric assays, have at best a sensitivity of 0.08 mU/litre, and a range of 0.5 to 200 mU/litre. A monoclonal immunochemiluminometric assay has a sensitivity of 0.004 mU/litre and a range of 0.6 to 200 mU/litre [268]. Similarly assays for thyroxine, IgG, insulin, digoxin, biotin and testosterone all compare favourably with other methods. Very small quantities of substances can now be analysed with only 500 attomoles of α_2 macroglobulin and 75 attomoles of ferritin being detected.

The alternative to using chemiluminescent labelled antibody or antigen, using enzyme labelled antibody and chemiluminescent substrate, may confer even greater sensitivity. As little as 100,000 molecule/ml (5 femtograms or 0.125 attomoles) of horseradish peroxidase are detectable using luciferin substrate [205], and thus the problems of labelling IgG are avoided, but luminol substrate was much less sensitive. A comparison between assays employing luminescent labelling and peroxidase labelling shows that the latter method may yield better results [270]. For example, insulin labelled with luminol in a competitive assay gives a range of 5-25 ng/litre, and using an HRP label, 4 ng/litre. HRP catalysed colorimetric analysis has also been compared with HRP chemiluminometric analysis using isoluminol for quantification of serum proteins [201], which showed an increased sensitivity of up to 95-fold for IgG, using a competitive binding assay. A comparison of a sandwich ELISA, using absorptiometric and chemiluminometric detection for HSV again using isoluminol, showed that the chemiluminescence based assay was 64-fold more sensitive, with 40 pfu/sample detected

versus 2,500 pfu, and when applied to clinical specimens, detected 15/17 specimens positive on culture versus 12/17 on ELISA [201]. However, no CSF specimens were positive [203, 202]. Chemiluminescence assays have similarly been used for other viruses, such as cytomegalovirus [201], and Venezuelan equine encephalomyelitis virus [178], with results equal to or better than RIA and absorptiometric analysis.

In the work presented later, therefore, two systems of chemiluminescence were to be compared. The system based on luminol was using the chemiluminescent material purely as a substrate for an enzyme catalysed reaction. In contrast, the system based on isoluminol was using the chemiluminescent material as the label.

E. Techniques for improving light output for isoluminol and luminol

The light output from HRP catalysed oxidation of cyclic diacylhydrazides tends to be short-lived and inefficient. Reactions can be enhanced by the addition of other compounds such as luciferin or other 6-hydroxybenzothiazole derivatives, but by far the best enhancement has been obtained with phenol derivatives, particularly p-iodophenol [248]. With the addition of this to luminol, light emission is increased by 2,500-fold, with not only greater intensity, but much more prolonged output over many minutes. For isoluminol, the enhancement was about 100-fold. Enhancement was pH dependent, the optimum being pH 8.5, with rapid tail off either side of pH 8 and 9, and enhancement was also dependent on p-iodophenol concentration. p-iodophenol inhibits haem catalysed light emission. The effectiveness of enhancement was not influenced by the solid phase, or the type of assay, but each assay required its own optimisation of conditions.

One factor affecting the enhancement is the purity of the luminescent reagent. Commercial luminol is a very heterogeneous material with up to 20 components, and purification methods are needed to ensure maximum enhancement [240]. Purified luminols give up to 100-fold improvement on unpurified preparations.

Both luminol and isoluminol are light and temperature sensitive. Light induces chemical changes to produce products that are inhibitory to enhanced luminescence, and luminol is thermally unstable, although usually high temperatures (> 200°C) are needed.

3. Detection Systems

Detection systems for light output can be simple or complex. At the simplest level a dark adapted eye can see luminescence in the reaction tube as a blue light, but large amounts of luminescent material are required, and for assay systems requiring sensitivity, the light output is usually not visible.

The simplest materials which can be used are photographic film [248], which results in a permanent, though only semi-quantitative record. Since multiple reactions can be recorded at one time, this however could be used for screening. Results are only useful for measuring quantities of substances in a known range, not for sensitive detection. X-ray plates have also been used [145], as the film is more sensitive to light than to gamma rays, and can be used for imaging immunoblots, but again results are only semi-quantitative.

For quantitative results, commercial equipment can be modified, including photometers, photomultipliers [205], fluorimeters and scintillation counters [270]. In recent years purpose built equipment has become available, and such luminometers are capable of measuring not only peak signals, but integrate signals over a range of times, measure slopes and end points. They have also the facility to add reagents to the photomultiplier chamber, for those reactions with immediate light responses. The main drawback is that multiple measurements can only be made sequentially, thus the mechanics of ensuring identical incubation times, temperature control, light control etc become cumbersome when large numbers of measurements have to be made.

SECTION 2

MATERIALS, METHODS AND RESULTS

Correction Statement

Dilutions throughout this section should be read as 1 in X, not 1:X as given.

Chapter 6: ASSESSMENT OF ISOLUMINOL AND LUMINOL BASED CHEMILUMINESCENCE

1. Materials and Methods

A. Setting up reagents for Isoluminol Chemiluminescence

i) Manufacture of Isoluminol Ester

The method of manufacture of the active isoluminol ester is taken from Wood et al.[282], with minor modifications as detailed. This method was followed for all subsequent manufacture of isoluminol labelled antibodies. All reactions were carried out in foil covered glassware which had been acetone cleaned and air dried. 10 mg of 6-N-(4-aminobutyl)-N-ethyl-isoluminol (ABEI) (Sigma, Poole, Dorset) was suspended in 3 ml of dimethyl formamide (DMF) (Sigma, Poole, Dorset), giving a concentration of 12 mM, and agitated until dispersed evenly. Then 25 mg of succinic anhydride (Sigma, Poole, Dorset) was dissolved in 1 ml of DMF at room temperature (RT), giving a concentration of 0.25 M, and 0.15 ml of this solution was mixed with the ABEI suspension with agitation, left at 4°C overnight, heated to 45°C for 3 hr, and then 60°C for 1 hr to completely dissolve the precipitate. 20 mg of N-hydroxy succinamide (NHS) (Sigma, Poole, Dorset) was dissolved in 1 ml of DMF at RT to give a concentration of 0.17 M, and 0.15 ml of this was mixed with the ABEI solution at RT. 28 mg of dicyclohexyl carbodiimide (DCCI) (Sigma, Poole, Dorset) was dissolved in 1 ml DMF at 60°C, to give a concentration of 0.14 M, and 0.6 ml of this added to the ABEI-NHS and left at RT overnight. This active ABEI ester was stored at 4°C in the dark until conjugation to protein.

ii) Conjugation of Isoluminol to protein

The method of conjugation of the active ABEI ester to antibody was taken from Wood et al. [282] and was applied to all the antibodies subsequently labelled, the only variation being made was the molar ratio of label to antibody in conjugation. For the initial studies, 0.5 ml of rabbit anti-HSV1 IgG (MacIntyre strain, Dakopatts, High Wycombe, Bucks) was diluted in 5ml 0.05 M Phosphate Buffered Saline solution (PBS) pH 8.0. To this, 0.2 ml of ABEI-NHS was added with mixing, and left at RT overnight to allow conjugation. The solution was transferred to visking tubing (BDH, Poole, Dorset), dialysed against PBS for 3 exchanges for 2 hr each and one overnight exchange to remove unbound label. The dialysed conjugate was aliquoted and stored at -35°C , being thawed only once before use.

iii) Optimisation of Chemiluminescence system

All reactions involving chemiluminescence were carried out using an LKB 1251 luminometer (LKB/Pharmacia, Milton Keynes). The reaction was initiated within the measurement chamber by an automatic dispenser which released measured aliquots of the source of $\text{O}^{\cdot -}$ radicals. Output was displayed both digitally on the luminometer and graphically using an Omniscrite chart recorder (Houston Instruments, Houston, Texas). Numerical values were taken from either peak readings or time integrated displays from the luminometer, and light output kinetics from the chart recorder.

a) Hematin concentration

100 μl of 1:1000 ABEI labelled rabbit anti-HSV1 IgG in 0.15 M NaCl was mixed with 0.5 ml hematin (Sigma, Poole, Dorset), at a range of concentrations from 0.1875-750 μM , and the luminescence initiated with 50 μl 18mM H_2O_2 in 0.33 M NaOH. Because of

the kinetics of the light response (a sudden flash and a rapid tail off - see figure 11), the peak light output value for each combination was plotted, and by using mixtures without labelled IgG and volume made up by NaCl, a signal to noise ratio was achieved.

b) Choice of O^- producer

The hematin calibration curve was repeated replacing the H_2O_2 with 18 mM $NaBO_3$, and the average of 10 peak readings taken and plotted as before.

c) Response curve for labelled IgG

100 μ l aliquots of ABEI labelled rabbit anti-HSV1 IgG, diluted from 1:3625-1:130,000 in 0.15 M NaCl were mixed with 0.5 ml of 0.75 μ M hematin in 0.15 M NaCl and luminescence initiated by 50 μ l of 18 mM H_2O_2 . An average of 4 readings was made for each dilution and the peak light output plotted.

iv) Calibration of Isoluminol ester base

The optimised concentrations were used to set up a calibration curve for the light output of the ABEI ester. 0-100 μ l of a $1:4 \times 10^6$ dilution of stock ABEI-NHS in 0.15 M NaCl was mixed with 0.5 ml 0.75 μ M hematin in 0.15 M NaCl and light output initiated as above. The total volume was made up to 0.65 ml with 0.15 M NaCl. This range of ABEI-NHS was chosen because it gave a light output within the range of detection of the luminometer. Light output was plotted against molar concentration of ABEI-NHS manufactured in experiment 1 A which had a concentration of 0.25 pmol/100 μ l (2.5 nM).

B. Assessment of commercial anti-HSV1 IgG labelling

i) Purification of IgG by Rivanol extraction and Calculation of the Protein content

The method of Rivanol (diamino acridine lactate) extraction was taken from the Handbook of Experimental Immunology [104], and is detailed in Appendix 2. 500 μ l of rabbit anti-HSV1 IgG was purified, and stored at 4°C until use.

The standard Biuret method for plasma protein estimation [1] was used to estimate the IgG content of the purified antibody solutions. Protein calibrant no.4 (ATAB, Warriner, Berks.) with a protein content of 52 g/l was used to construct a calibration curve and absorbance was measured at 555 nm in a Uvikon 810 spectrophotometer (Kontron Instruments, Watford).

ii) Assessment of optimum Isoluminol to IgG ratio

Conjugation of ABEI-NHS to rabbit anti-HSV1 IgG was performed as in 1 A with the volume of purified IgG being varied from 0.05-0.5 ml (0.5-5 mg) and the reaction volume remaining constant by varying the PBS volume. After dialysis the final solutions were then diluted by 1:500-1:5000 to achieve an equal IgG final concentration in PBS, and a 100 μ l of each was read in the luminometer. An average of 10 peak light output readings was made for each conjugate, and the specific activity plotted by converting the light output value into moles of ABEI-NHS and dividing by the molar values for IgG.

iii) Immunoprecipitation of labelled IgG

20 ul of the optimum ABEI IgG was incubated at RT overnight with an excess (40 ul) goat anti-rabbit IgG (ATAB, Winnersh, Berks.) in a total volume of 200 ul made up with PBS. The mixture was centrifuged at 8000 g in an Eppendorf microfuge 5413 (source to enter) for 45 min, the supernatant decanted and 100 ul of a 1:5000 dilution in PBS counted in the luminometer. A control of ABEI IgG identically treated, but without the goat antisera, was run in parallel.

C. Assessment of immunological function of the Isoluminol conjugated IgG

In these experiments preservation of the immunological function of the labelled IgG was assessed by coupling HSV antigen to a solid phase, and incubating labelled IgG with the bound antigen. An initial attempt at eluting IgG bound to the column was also made with the intention of using this affinity purified antibody in the assay system.

i) Estimation of protein concentration of HSV1 antigen

The protein concentration of commercial HSV1 antigen (Behring Diagnostics, Hoescht UK Ltd, Hounslow, Middlesx) was estimated using the Biuret method.

ii) Coupling of commercial HSV1 antigen to Cyanogen Bromide activated Sepharose 4B

The method was taken from Affinity Chromatography, Principles and Methods, an application guide provided by Pharmacia [2]. This method was followed in all subsequent couplings with only minor variations detailed where appropriate in the text.

1 g freeze-dried Cyanogen Bromide activated Sepharose 4B (CNBr-Seph) was activated by mixing with 10 ml 1mM HCl, left for 15 min to swell and then transferred to a sintered glass filter, where, under vacuum, the gel was washed with 200 ml 1 mM HCl in several aliquots. 1 ml of HSV1 antigen was mixed with 6 ml coupling buffer, consisting of 0.1 M NaHCO_3 and 0.1 M Na_2CO_3 in 0.5 M NaCl mixed to a pH of 8.35. The gel was vacuum washed with 5 ml coupling buffer and immediately transferred to the HSV1 antigen solution where it was mixed by rocking for 2 hr at RT. The remaining active groups on the CNBr-Seph were blocked with 0.2 M glycine in coupling buffer which was left with occasional mixing at 4°C for 16 hr. The blocked gel was vacuum washed on the sintered glass filter alternately with coupling buffer and 0.1 M acetate buffer in 0.5 M NaCl pH 5.2. Washing took place 4-5 times to remove protein ionically bound,

the final wash being coupling buffer. The gel was finally suspended in 7 ml coupling buffer, a few drops of 1% Sodium Azide added as a bacteriostatic agent, and the suspension stored at 4°C for immediate use.

iii) Binding of labelled IgG to Sepharose coupled HSV1 antigen

Incubation of labelled antibody and coupled antigen would enable the proportion of specific labelled and immunologically functional antibody to be estimated by comparing the free to bound ratio. The use of a range of dilutions of labelled antibody would ensure that the antigen was saturated and thus optimum proportions obtained for later attempted elution of bound antibody.

100 ul ABEI labelled rabbit anti-HSV1 IgG diluted from neat to 1:5,000 in PBS pH 8 was incubated with 20 ul CNBr-Seph-HSV1 for 2 hr at RT with continual slow mixing. Each mixture was then centrifuged for 5 min, the supernatant eluted, the gel washed 5 times with PBS and the supernatants read immediately in the luminometer, using the previously optimised reagents. Appropriate dilutions were made for those supernatants whose readings initially overloaded the photomultiplier. Finally the gels were read, all the values corrected for background, and the final results expressed as a percentage of gel counts over the total counts added.

iv) Affinity purification of labelled IgG

Two methods of producing affinity purified labelled IgG were attempted. In the first, pre-labelled IgG was bound to a CNBr-Seph-HSV1 column and the second unlabelled IgG was bound and labelled in-situ; the latter method was tried to improve the yield of immunologically active labelled IgG by covering the Fab site. In addition a modification of the binding of the antigen to the Seph-4B column was made by pretreating the antigen with 8M Urea/Triton X100 to improve the exposure of epitopes [81].

50 ul rabbit N-IgG (Dakopatts, High Wycombe, Bucks) and anti-HSV1 IgG were diluted to equal the IgG content of labelled IgG and incubated with 100 ul of CNBr-Seph-HSV1 for 30 min at RT. After washing x 5 with PBS pH 8.0, and centrifugation, 100 ul ABEI-NHS was added in 1.5 ml PBS and incubated at 4°C for 72 hr with mixing before washing excess. 50 ul of rabbit N-IgG was labelled with ABEI-NHS as before, and together with labelled anti-HSV1 IgG were both incubated with CNBr-Seph-HSV1 for 30 min at RT. After washing all the gels were then incubated with 1.5 ml of increasing concentrations of NaSCN (10^{-6} - 4 M), mixed for 15 min at RT, centrifuged and the supernatants eluted. In between steps the gels were washed with PBS. 100 ul 1:100 in PBS of each supernatant was counted in the luminometer, using standard reagents.

1 ml control HSV antigen (Behring diagnostics, Hoescht UK Ltd., Hounslow, Middsx.) and 1 ml HSV1 antigen were each pretreated with 10 ml 8 M Urea/1% Triton X100 for 1 hr at RT before coupling to sepharose as standard. Antibodies, both labelled and unlabelled, were incubated with sepharose bound antigen, the NaSCN elution and supernatant counting performed as above.

v) Dialysis of eluants from affinity purification

Because eluants from the antibodies labelled in-situ may have contained free isoluminol each of the eluants from the previous experiment was dialysed using visking tubing against PBS for 24 hr with 4 changes. 100 ul of each eluant was then counted in the luminometer, initially at a 1:100 dilution, but because the counts were so low, the eluants were counted undiluted using standard reagents.

D. Setting up reagents for luminol chemiluminescence.

i) Manufacture of purified luminol base

Enhancement of light output of luminol by purification was performed by the methods described [240]. All reactions were performed in foil covered glassware and incubations took place in the dark. 3.25 g 3-aminophthalhydrazide (luminol) (Aldrich, Gillingham, Dorset) was dissolved in 18 ml of 5% NaOH at RT until saturation was reached. After filtration through a 0.2 μ m Millipore filter (Millipore (UK) Ltd., Watford) to remove undissolved luminol, the solution was cooled to 4°C and kept for 16 hr whilst crystallisation took place. After drying on Whatman No 1 filter paper (Whatman, Maidstone, Kent), the crystals were redissolved at RT in a minimal volume of 5% NaOH (10 ml), refiltered through the Millipore filter and the solution cooled to 4°C for 72 hr whilst crystallisation occurred. The crystals were washed on Whatman filter paper with ice-cold 5% NaOH, and left to dry. The final yield was 1.7 g of white crystals. Luminol base was prepared by dissolving the purified luminol in distilled H₂O to a concentration of 100 mg/ml, then adding drops of concentrated HCl until no further precipitation formed. The supernatant was discarded and the crystals dried at RT. The final yield was 0.5 g and the purified luminol base stored in a stoppered bottle at 4°C in the dark.

ii) Initial assessment of Iodophenol enhancement

The method of enhancement using iodophenol was modified from that described by Thorpe et al. [248]. Stock p-Iodophenol (Aldrich, Gillingham, Dorset) was made by dissolving 17 mg in 0.5 ml dimethyl sulphoxide (DMSO) (BDH, Poole, Dorset), and making up to 5 ml with 0.05 M PBS pH 7.4. Working solutions were diluted further in PBS. Stock luminol solution was made by dissolving 10 mg purified luminol base in 0.5 ml 0.33 M NaOH and making up to 5 ml with PBS. Working solutions were diluted

further in PBS. To catalyse the reaction, goat anti-rabbit IgG HRP conjugate (Miles-Yeda, ICN Biomedicals Ltd, High Wycombe, Bucks.) was diluted as appropriate for use in PBS. 20 ul of appropriate dilutions of iodophenol, luminol and HRP conjugate were mixed in 300 ul of PBS and luminescence initiated using 50 ul 18 mM H_2O_2 in 0.33 M NaOH.

The kinetics of light output from luminol differed from that produced by isoluminol, in that there was an initial flash followed by a prolonged plateau phase whose shape varied depending on the reagent proportions (see Figure 11 later). Both plateau phase and peak light output were measured, but only the plateau phase showed any relationship with reagent concentrations. Unless otherwise indicated, for luminol the 120 s integrated area curves were used. Light output was then measured using a variety of luminol and HRP concentrations, and once these were optimised, a range of iodophenol dilutions was then applied to establish its optimum.

iii) Choice of O^-

As with isoluminol based chemiluminescence, the choice of O^- source was H_2O_2 or NaBO_3 . 100 ul of dilutions of goat anti-rabbit IgG HRP conjugate were mixed with 100 ul 1:20 stock iodophenol and 100 ul 1:50 stock purified luminol base.

Chemiluminescence was initiated with 30 ul of dilutions of either of the O^- sources. In this experiment the 40 s integrated time was plotted for each of the sources.

iv) Calibration curve for iodophenol enhanced luminol chemiluminescence

20 ul aliquots of 1:4 iodophenol, 1:2000 luminol and 1:100-1:50,000 goat anti-rabbit HRP conjugate and rabbit anti-goat IgG HRP conjugate (Dakopatts, High Wycombe, Bucks.) were all diluted in 400 ul PBS and the reaction initiated with H_2O_2 as above. The 120 s integrated count was recorded with values corrected for background.

v) Reagent stability

a) Effect of Time

Reactions mixes were as for section iii) with standard 1:2000 goat anti-rabbit IgG HRP conjugate being used. The mixture was incubated at RT for up to 1 hr before initiation of chemiluminescence, and a parallel was also incubated, but with the iodophenol added immediately prior to reading. A 40 s integrated time result was plotted.

b) Effect of Temperature

The reaction mix was made up as for a) but were kept at 4°C or RT or combinations for up to 75 min and chemiluminescence initiated.

2. Results

A. Isoluminol based Chemiluminescence

i) Optimisation of Chemiluminescence

a) Hematin concentration

Figure 4 shows that 0.75 μM of hematin gave the best signal:noise at 3.5:1. Note that marked quenching of light output occurred at higher concentrations, the solution being a dark red colour.

b) Choice of O^- producer

The curve for NaBO_3 paralleled that for H_2O_2 , but although light output was greater, because of higher background noise, the signal:noise was invariably worse, and so H_2O_2 was the choice.

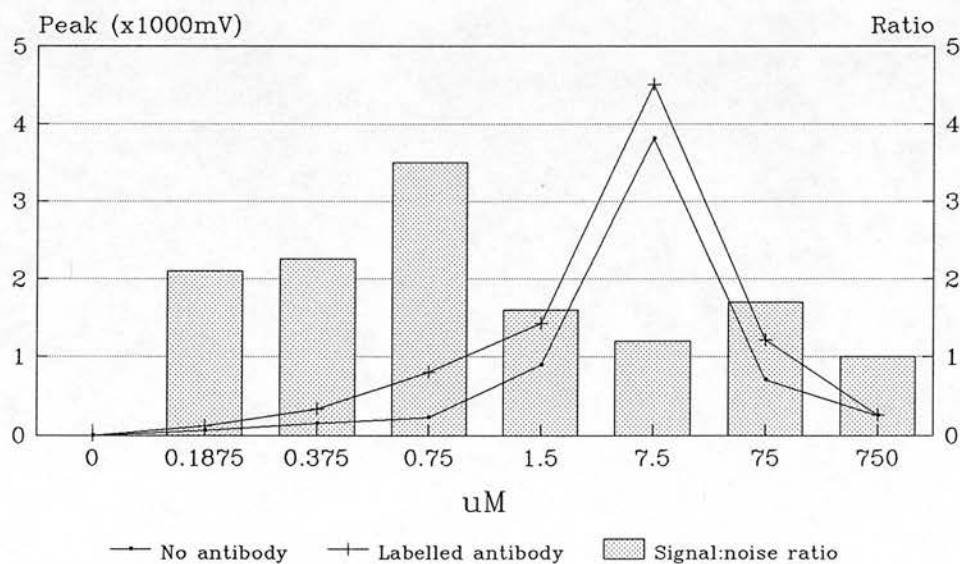


Figure 4: Optimisation curve for Hematin catalysed luminescence

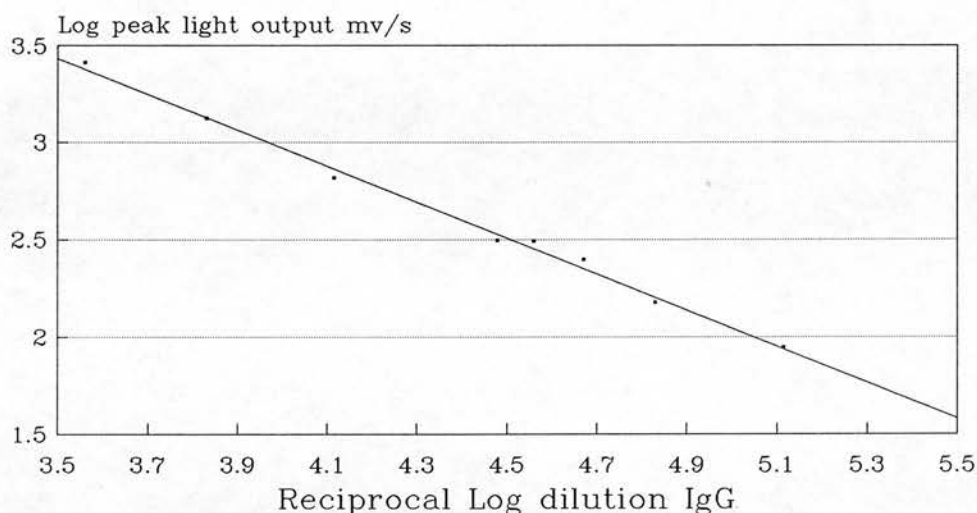


Figure 5 : Calibration curve for Isoluminol labelled IgG

c) Response curve of labelled IgG

Figure 5 shows a straight line log:log relationship over the range of light output detection range of the luminometer. Sensitivity was high, with 1:130,000 dilution showing counts > 100% above background. This dilution of labelled IgG was equivalent to 4.6×10^{-15} mol.

All subsequent experiments were conducted using 0.5 ml of 0.75 μ M hematin and 50 μ l of 18 mM H_2O_2 unless otherwise indicated.

ii) Calibration of isoluminol ester base

Figure 6 shows the peak light output plotted against the absolute amount of ABEI-NHS in moles. The graph shows a linear relationship with a slope of 4.5×10^{12} volts/s/mol ABEI-NHS.

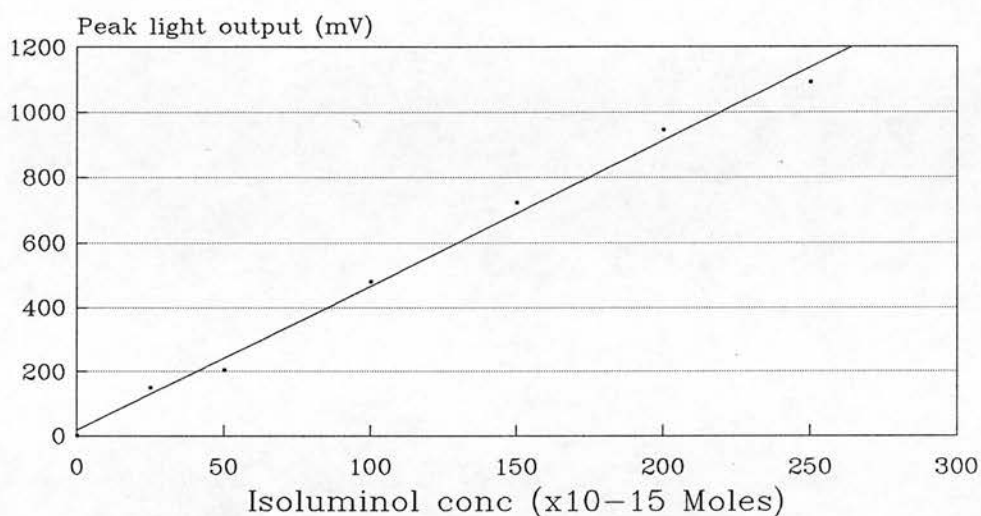


Figure 6 : Calibration curve for isoluminol light output

iii) Assessment of IgG labelling

a) Optimum Isoluminol:IgG ratio

The IgG content of purified commercial rabbit anti-HSV1 IgG was 10 g/l. Table 19 shows the specific activity of various combinations of ratios of isoluminol ester to IgG.

IgG Vol. (mls)	Peak ht.* (mV)	Isoluminol conc. (x10 ⁻¹⁵ moles)	Specific activity
0.05	568	126	1.14
0.10	370	82	0.74
0.25	415	92	0.84
0.50	315	70	0.64

* - Identical IgG ammounts from each combination
(110 x 10⁻¹⁵ moles)

TABLE 19: Resultant specific activity from varying Isoluminol and IgG conjugation proportions

The best mix was 0.05 ml antibody to 200 ul isoluminol ester. This gave a specific activity of 1.14, thus indicating that on average one molecule of isoluminol label was attached to each molecule of IgG. The lower the isoluminol:IgG ratio, the worse the specific activity.

b) Immunoprecipitation of labelled IgG

Immunoprecipitating labelled IgG with goat anti-rabbit antiserum resulted in a 74% reduction in chemiluminescence output from the supernatant, suggesting there was predominantly labelled IgG present in the solution.

iv) Assessment of Immunological function of labelled IgG

a) Binding of labelled IgG to Sepharose coupled HSV1 antigen

The total protein content of the antigen was 19 g/l and appropriate volumes used for coupling to the seph-4B. Table 20 shows that labelled antibody never achieved saturation of antigen, with the average proportion of labelled IgG immunoreactive with HSV1 at only 1.7%, with at best 4.2% being bound.

b) Affinity purification of labelled IgG

Attempted elution was completely unsuccessful. There was no difference between control labelled antibody and anti-HSV1 labelled antibody with both seph-4B-HSV1 columns and U/T control and HSV1 treated antigen seph-4B columns at any concentration of NaSCN, irrespective of whether the antibody was incubated prelabelled or after attachment to the column. Prelabelled antibody supernatant counts were overall much lower than when labelling was attempted after binding to the column, particularly in eluants from NaSCN concentrations above 1 M.

IgG incubated ($\times 10^{-15}$ moles)	Supernatant Peak (mV)	Gel Peak (mV)	%age bound
55000	171600	4621	2.6
27500	57025	1044	1.8
11000	32400	659	2.0
7330	23359	352	1.5
5500	19590	249	1.3
3670	12324	80	0.6
2750	8441	102	1.2
2200	6962	302	4.2
1100	3483	10	0.3
550	1510	21	1.4
275	1335	0	0
138	891	0	0
110	476	0	0
55	209	0	0
22	91	0	0
11	26	0	0

TABLE 20: Binding of labelled anti-HSV1 IgG to an HSV1 coated sepharose 4B column.

c) Dialysis of eluants from affinity purification

Dialysis of eluants from those columns where isoluminol labelling occurred after antibody binding showed a large reduction in chemiluminescent activity by about 95%. There was no difference between normal IgG and anti-HSV1 IgG in either control or HSV1 antigen columns at any of the NaSCN dilutions, and no specific increase in chemiluminescence above 1 M concentrations.

B. Luminol based Chemiluminescence

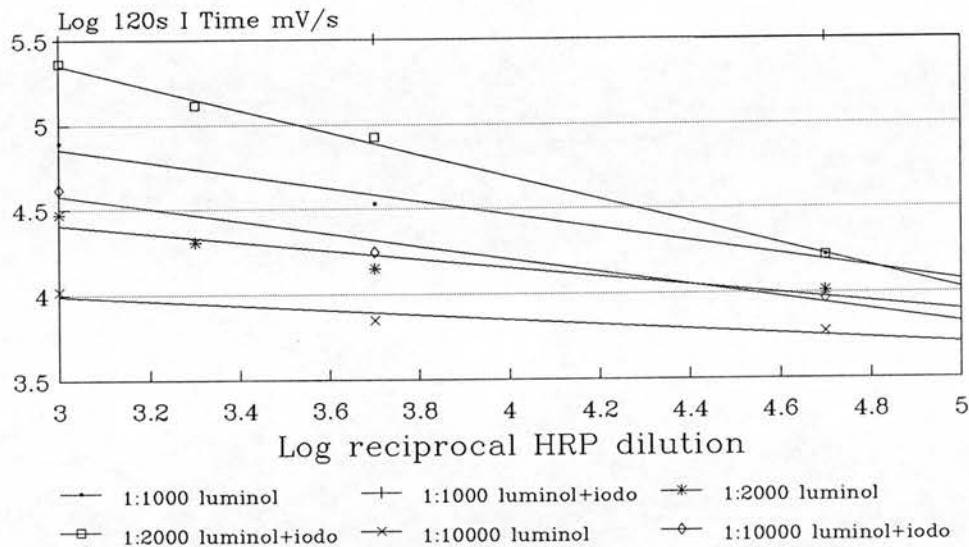
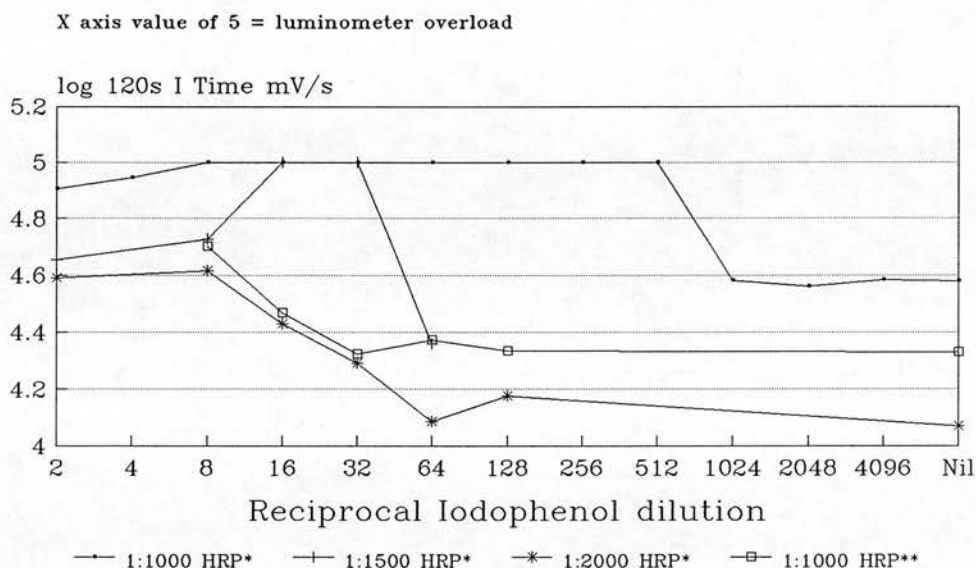


Figure 7 : Iodophenol enhancement of Luminol chemiluminescence

i) Iodophenol enhancement

Figure 7 shows that iodophenol enhancement is dependent on concentration of both luminol and the HRP conjugate, with luminol dilutions > 1:10,000 giving no response and HRP enhancement being more marked at dilutions < 1:50,000. High concentrations of luminol overload the system as a result of the initial flash and not the plateau phase. A 1:2000 dilution of luminol base was the best substrate concentration for iodophenol enhancement.



* - 1:2000 luminol, ** - 1:4000 luminol

Figure 8 : Optimisation of Iodophenol concentration

Figure 8 shows that enhancement was not only dependent on the concentration of luminol and HRP, but also varied depending on the concentration of iodophenol. A 1:8 dilution of iodophenol was optimum for a 1:4000 dilution of luminol and a 1:1000 dilution of HRP, with higher concentrations of iodophenol resulting in less enhancement. As the concentration of luminol was increased to 1:2000, which was the best initial working concentration, the luminometer system overloaded below 1:4 dilutions of iodophenol because of the initial flash, so the optimum measurable was 1:4. As the HRP concentration was reduced, the optimum dilution of iodophenol appeared to be between 1:4 and 1:8. However the maximum recordable enhancement for iodophenol was only 4-fold, somewhat less than the 100-fold enhancement reported in the literature [248].

ii) Choice of O⁻ source

Figure 9a shows the curves for H₂O₂ dilutions. A 1:2 dilution of stock 18 mM solution gave better responses at higher concentrations, but little improvement at lower concentrations of HRP conjugates. Below a 1:4 dilution, the response was much poorer.

Figure 9b shows curves for NaBO₃ dilutions. A 1:4 dilution of stock gave the best range of sensitivities of all the dilutions, and also gave a smoother curve than H₂O₂, but the background counts were all much increased as with isoluminol and the initial spike was higher. Overall the sensitivity was less than H₂O₂ at either stock or 1:2 dilutions.

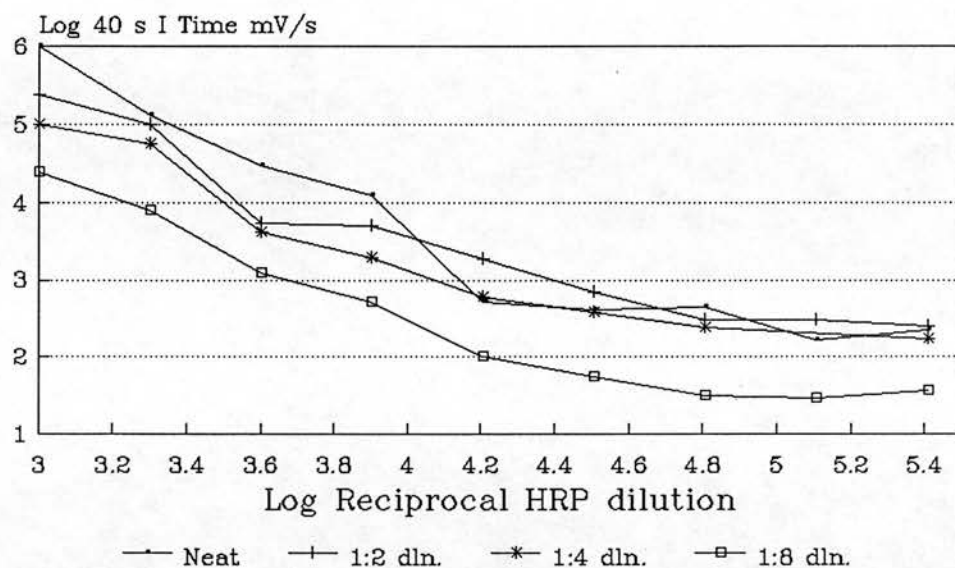


Figure 9a : Optimisation of Hydrogen Peroxide concentration for Luminescence

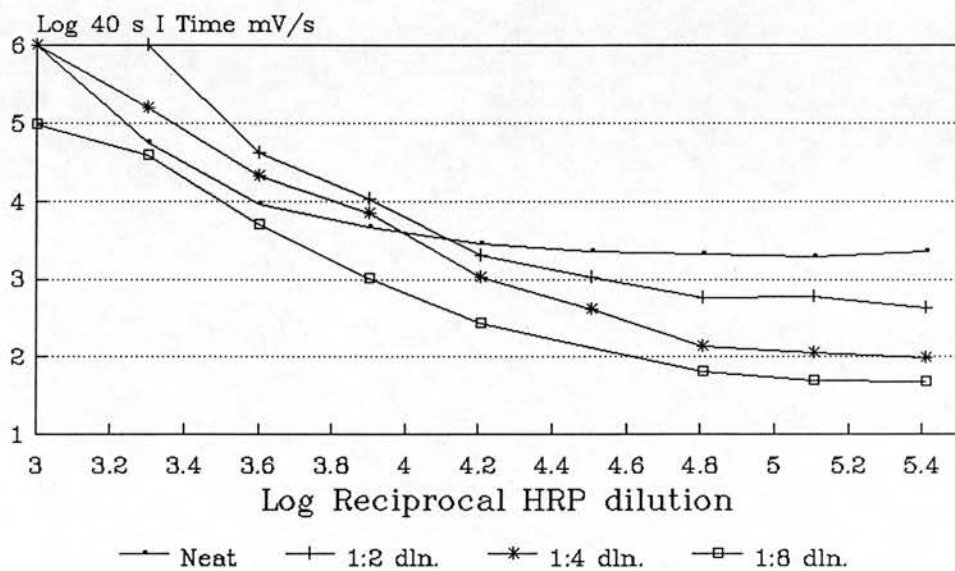


Figure 9b : Optimisation of Sodium Perborate concentration for Luminescence

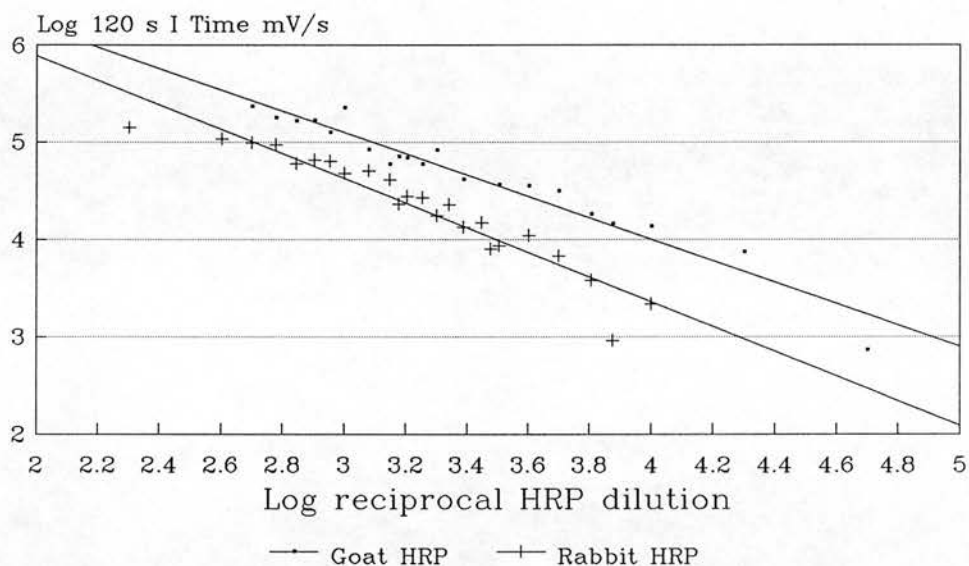


Figure 10 : Calibration curve for iodophenol enhanced luminol luminescence

iii) Calibration curve for iodophenol enhanced luminol chemiluminescence

Figure 10 shows that there was a linear relationship between light output and iodophenol enhanced luminol based HRP conjugate catalysed chemiluminescence. However the sensitivity of the luminescence was poor, with dilutions $> 1:6400$ (1.8×10^{-13} moles HRP conjugated IgG) giving $< 100\%$ counts above background. This would appear not to be sensitive enough for a sandwich assay.

iv) Reagent stability

a) Effect of Time

After 1 hr at RT without iodophenol in the mix there was a 40% reduction in light output, but with iodophenol an 88% reduction occurred, indicating that iodophenol inhibited light output and thus should only be added at the last moment.

b) Effect of Temperature

At 4°C there was no reduction in light output after 30 min, and only 10% after 75 min, compared with RT values of 35% and 61% respectively. If the luminol was kept at 4°C and the HRP at RT, the reduction was 30%, indicating that 50% of the reduction was due to instability of luminol and 50% the HRP at the dilutions used.

3. Discussion

Commercial rabbit anti-HSV1 IgG was successfully labelled with isoluminol to produce an immunologically active antibody, with a specific activity of 1.14, but a relatively low yield at 1.7% of the total IgG labelled.* The response curve of the isoluminol labelled IgG was shown to be a linear relationship, and thus suitable for calibration purposes, with a sensitivity of 4.6×10^{-15} moles of IgG. Attempts at affinity purification of the labelled IgG were all unsuccessful, irrespective of the method of labelling. The reasons for this were not clear, although the possibilities included the inactivation of the label by the chaotropic agent used or the failure of the chaotropic agent to separate high affinity antibody from the column. This latter option seemed unlikely to account for a total failure, as one would have expected a commercial polyclonal antibody to have contained a wide range of antibody affinities.

Despite the evidence from the literature indicating the higher sensitivity of iodophenol enhanced luminol luminescence, the results here were disappointing, with poor enhancement (only 4-fold versus 100-fold in the literature) and consequently poor sensitivity compared to isoluminol at 1.8×10^{-13} moles of IgG, a factor of 100-fold less sensitive. The kinetics of light output differed between the 2 substrates, with isoluminol giving a sudden short lived predictable peak light output and luminol giving an unpredictable initial flash followed by a long lived predictable plateau phase (Figure 11). These kinetics of the luminol chemiluminescence appeared mainly to blame for its poor performance. The initial flash did not result from impurities in the luminol or relate to HRP conjugate concentration, but appeared to be due to an instability in the reaction system. In addition, each reaction mix for luminol required individual optimisation. As a result, isoluminol labelling of antibodies was the preferred method.

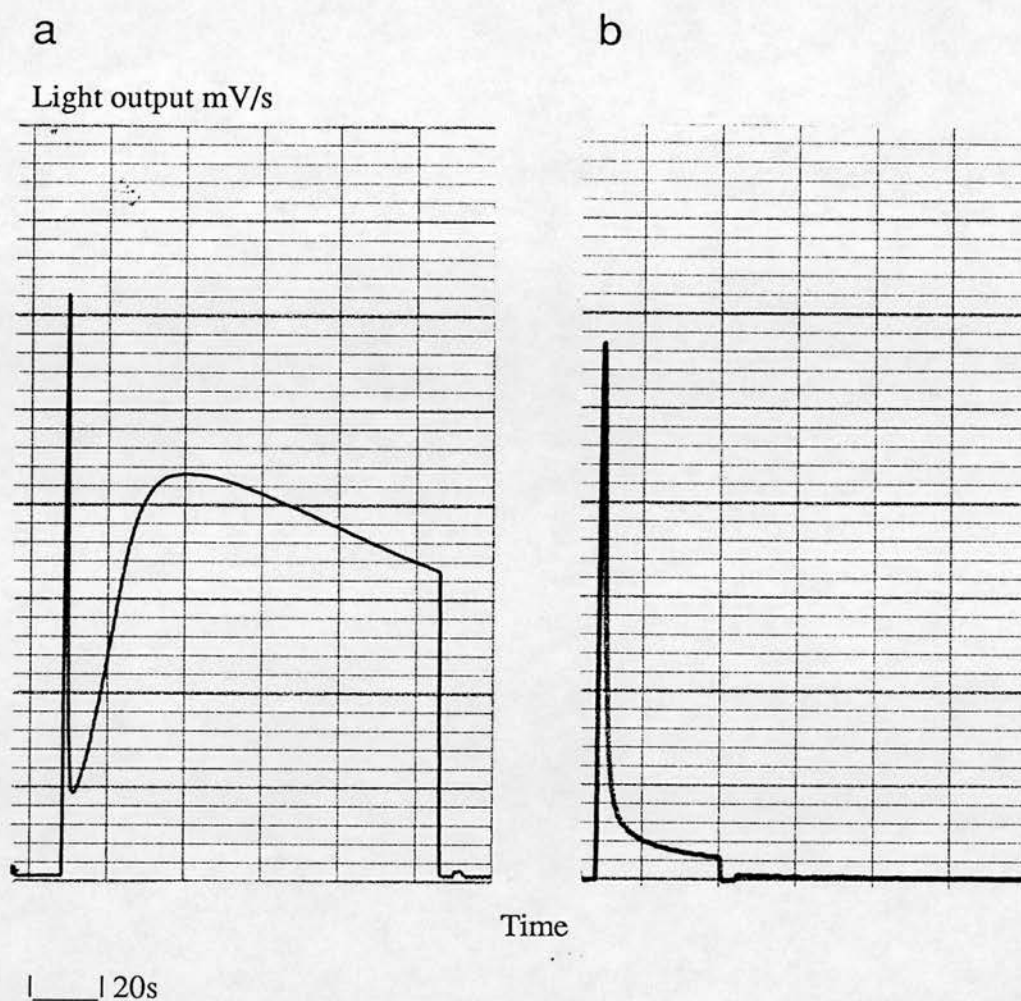


Figure 11 : Kinetics of Chemiluminescence

a) Luminol - p-Iodophenol enhanced

b) Isoluminol

Temperature and incubation time were both shown to be important in influencing light output, so all subsequent reactions were carried out with reagents kept at 4°C and reagent mixing times kept as short as possible before initiating chemiluminescence.

* This figure of 1.7% represents the proportion of immunologically functional labelled IgG compared to the total known to be labelled. The poor yield may have resulted from the binding of isoluminol close to, or on, the Fab site of the IgG molecule, thus sterically hindering immunological function. The Fab site would have to contain a disproportionate number of isoluminol conjugation sites for this to happen.

CHAPTER 7: ISOLUMINOL LABELLING OF ANTIBODIES FOR USE IN AN ASSAY SYSTEM

In this section a range of antibodies of potential use in an assay system were labelled, and the effectiveness of labelling and immunoreactivity assessed using a non-commercial HSV1 strain whose viral content was known and thus the sensitivity of the antibodies could be determined.

1. Materials and methods

A. Use of HSV Kos in affinity purification of labelled commercial anti-HSV1 IgG

i) Use of NaSCN and NaCl to elute antibody

HSV Kos was gifted by Dr. Robert Honess from Mill Hill MRC research laboratories. Unpurified virus grown in vero cells and vero cell controls were used. The whole virus preparation was initially disrupted by sonication and solubilised using 1% Triton X100, with some also being disrupted by 8M Urea for particular antibody studies. The treated virus was then centrifuged at 8000g in an Eppendorf microfuge 5413 for 5 mins to clear cell debris, and the supernatant virus count estimated at 10^{10} infected cell products per ml (ICP/ml). The total protein content of the supernatant was 50g/l by biuret method. The vero cell control was treated identically.

Coupling of HSV Kos and vero cell control to Seph-4B was performed as in Chapter 6. Preliminary work showed that 8M urea treatment destroyed the immunoreactivity with the commercial antibody, so this was omitted. Incubation of 100ul gel with 100ul isoluminol labelled commercial rabbit anti-HSV1 IgG, serial elution of antibody with NaSCN and luminescence of eluants were all performed as in Chapter 6. In addition, the use of NaCl of increasing molarity as a chaotropic agent was also attempted. Counts

were corrected for background and expressed as a percentage of the total added.

Because the counts added for the NaSCN elution gave high eluant values which needed dilution for counting, a 1:5 dilution of the labelled IgG was used for the NaCl stage.

ii) Effect of NaSCN on chemiluminescence

100 ul of NaSCN concentrations from 10^{-6} -4 M were mixed with 100 ul 1:10 isoluminol labelled IgG made up to a total volume of 600 ul with PBS and the chemiluminescence initiated as standard.

iii) Assessment of IgG content of eluants by Immunoblotting.

25 ul of eluants from the affinity elution were air dried onto nitrocellulose membranes (Sartorius Ltd, Epsom, Surrey). The membrane was blocked with 1% bovine albumin (Sigma, Poole, Dorset) in 0.15 M NaCl for 1 hr, washed x 5 with distilled water, incubated with 50 ul goat anti-rabbit IgG HRP conjugate (Dako, High Wycombe, Bucks.) in 50 ml 0.1% albumin in 0.15 M NaCl for 1 hr, washed again, and then visualised with 10 mg 3-amino-9-ethylcarbazole (Sigma, Poole, Dorset) in 6 ml methanol and 44 ml 0.02 M acetate buffer pH 5.1.

iv) Viral content of HSV Kos preparation

The viral content of the HSV Kos preparation had been verified by Dr. Honess in his laboratory using standard techniques, and the details of this will not be described further.

B. Labelling and purification of Hyper-Immune rabbit antisera alpha-T2.

i) Use of pH to elute antibody

Two polyclonal hyper-immune rabbit anti-HSV1 IgG antibodies, alpha-T1 and alpha-T2, together with a pre-immune control serum were gifted by Dr. Sandy Buchan from the University of Birmingham. The IgG content of the antisera was estimated, after IgG purification, by modified rocket method [152] and labelling of the antibodies with isoluminol performed as in chapter 6. Incubation of labelled control antibody and alpha-T2 (which had the highest IgG content) with HSV Kos or vero cell control Seph-4B were performed as standard before elution of antibody by washing first with 0.05% Tween 20, then incubating the gel with 1 ml 0.1 M Glycine in 0.2 M HCl pH 2.7 for 8 min, the supernatant removed, neutralised with solid Tris to pH 9.0, and then 100 ul counted as before. The effect of acid treatment and neutralisation on chemiluminescence was first assessed, in view of the effect of NaSCN, by incubating aliquots of ABEI-NHS with glycine/HCl for a range of times before counting, and also counting after neutralisation, both immediately and after a delay of 30 min.

ii) Assessment of IgG content of eluants by immunoblotting

The procedure was identical to that followed above (section A iii).

C. Labelling and purification of a commercial monoclonal anti-HSV1 IgG

i) Labelling of antibody, Sephadex chromatography and assessment of specific activity

Dialysis of labelled antibody to remove unbound isoluminol was time consuming, so after labelling antibody as in Chapter 6, the dialysis step was replaced by a separation stage using sephadex chromatography. A 27 cm column of sephadex G-100 (Pharmacia, Milton Keynes, Bucks.) was thoroughly washed with 0.05 M PBS, and an ISCO UV detector, automatic fraction collector and chart recorder (ISCO, Lincoln, Nebraska) were calibrated and stabilised at a pump flow of 2 units, the fractions set at 0.5 ml. 1.2 ml of freshly prepared isoluminol labelled antibody was applied to the top of the column, and the fractions collected and marked on the chart recorder. The chart recorder was calibrated using 2 standard IgG solutions (0.15 and 0.3 g/l). Confirmation of the IgG content of the fractions was performed by immunoblotting as above (section A iii), with the use of goat anti-mouse IgG (Cappel laboratories, Cochranville, P.A., USA) and rabbit anti-goat IgG HRP conjugate, and the IgG value obtained from the calibration on the chart recorder. Chemiluminescence of each fraction was performed as standard on 100 ul of 1:10 or 1:100 dilutions. The specific activity was calculated by using the calibration curve for isoluminol in chapter 6.

ii) Affinity purification of labelled IgG using pH and NaSCN

The immunoreactivity of the labelled antibody, and an indication of the affinity was assessed by pH elution from the antigen coated seph-4B column. Preliminary experiments suggested that the monoclonal was directed against structural, non-envelope protein, and that pre-treatment of the antigen with 8M urea was necessary before coating the gel. 10 ul of labelled antibody from the optimum fractions (9 and 10 pooled) was incubated with 200 ul of control or HSV Kos seph-4B, with a minor modification, in that a mini-column of gel was made by loading a pasteur pipette stoppered with glass wool.

Centrifugation steps were thus eliminated. The column was washed with 0.05 M PBS, then 0.2 M glycine/HCl pH 2.7, and the eluant neutralised with solid tris. Washings eluants and the gel were all counted with standard chemiluminescence reagents.

In parallel, increasing molarities of NaSCN were used to elute antibody from identical control and HSV Kos columns, and the eluants immunoblotted for IgG content as above (section A iii) as well as chemiluminescence counted (since NaSCN inhibited chemiluminescence, immunoblots were needed to show successful elution).

D. Labelling and purification of human anti-HSV1 IgG

A serum from a survivor of HSVE with high titre of anti-HSV1 antibody (>1:32,000) was purified for IgG, labelled with isoluminol, separated by sephadex chromatography, IgG content estimated, chemiluminescence performed and specific activity of the optimum fraction calculated as above (section C i). Affinity elution of labelled IgG using Tween 20, pH and NaSCN from an HSV Kos column was performed as above (section C ii).

2. Results

A. Use of HSV Kos in affinity purification of commercial anti-HSV1 IgG

i) Use of NaSCN and NaCl to elute antibody

Table 21 shows that with NaSCN there appeared not only to be poor elution of antibody with chemiluminescent activity, but the counts dramatically dropped with the higher molarities. 6% of the added counts appeared to be unaccounted for with the HSV Kos column, whereas all the counts in the control column tallied.

NaSCN [M]	Control antigen Peak (mV/s) (%)	HSV1 antigen Peak (mV/s) (%)
Added	160000 (100.0)	160000 (100.0)
Nil	147600 (92.0)	136500 (85.3)
10 ⁻⁶	4500 (2.8)	4335 (2.7)
10 ⁻⁴	2865 (1.8)	2895 (1.8)
10 ⁻¹	3675 (2.3)	4875 (3.0)
1	465 (0.3)	855 (0.5)
4	240 (0.15)	765 (0.5)
Seph	0 (0.0)	0 (0.0)
Total	159345 (99.6)	150225 (94.0)
Unacc	655 (0.4)	9775 (6.0)

Unacc - chemiluminescence unaccounted for
Seph - sepharose 4B

TABLE 21: Elution of Isoluminol labelled anti-HSV1 IgG from antigen coated sepharose 4B column using NaSCN.

Table 22 shows that with NaCl, all the added counts were accounted for, and 3 M NaCl eluted 8.3% of the added counts to the HSV Kos column and 4% for the control column, indicating 4.3% specific IgG eluted.

NaCl [M]	Control antigen Peak (mV/s) (%)	HSV1 antigen Peak (mV/s) (%)
Added	30000 (100.0)	30000 (100.0)
Nil	27465 (91.5)	26070 (87.0)
0.15	0 (0.0)	0 (0.0)
0.5	660 (2.2)	420 (1.4)
1.0	285 (0.9)	510 (1.7)
1.5	210 (0.7)	345 (1.2)
3.0	1260 (4.0)	2475 (8.3)
Seph	0 (0.0)	0 (0.0)
Total	29880 (99.6)	29820 (99.4)
Unacc	120 (0.4)	180 (0.6)

Unacc - chemiluminescence unaccounted for
Seph - sepharose 4B

TABLE 22: Elution of Isoluminol labelled anti-HSV1 IgG from an antigen coated sepharose 4B column using NaCl

Thus there appeared to be a discrepancy in the results of NaSCN elution with respect to the chemiluminescence counts detected, the reason for which became apparent below.

ii) Effect of NaSCN on chemiluminescence

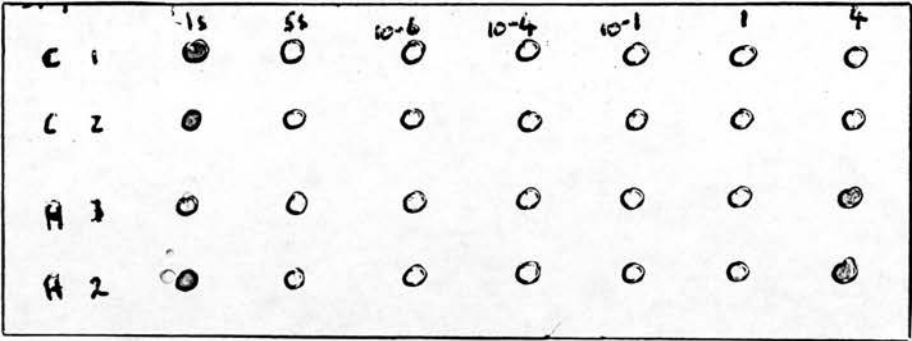
Table 23 shows that NaSCN inhibited chemiluminescence significantly above 0.1 M, and so the apparent poor yield from affinity purification may have been due to this effect. This inhibition was permanent, and not due to active interference, since in chapter 6 dialysis of eluants did not show recovery of chemiluminescent response in the high molarity range.

NaSCN [M]	Peak output (mV/s)	% inhibition
Nil	802	0
10 ⁻⁶	680	15
10 ⁻⁴	708	12
10 ⁻¹	584	27
1	210	74
4	71	91

TABLE 23: Inhibition of chemiluminescence by NaSCN

iii) Assessment of IgG content of NaSCN eluants by immunoblotting

Figure 12 shows that the 4 M NaSCN eluants from the HSV Kos column contained significant IgG staining, confirming that high affinity antibody was present whose chemiluminescence was inhibited by the NaSCN. Apart from the initial washings containing unbound IgG there was little or no significant staining in any other fraction.



1s, 2s - First and fifth washings

10⁻⁶, 10⁻⁴, 10⁻¹, 1, 4 - NaSCN Molarity

C, H - Control and Herpes Simplex columns

Figure 12 : Immunoblots of Isoluminol labelled antibody eluted from control and HSV Kos sepharose-4B columns with NaSCN

B. Labelling and purification of Hyper-Immune rabbit antisera alpha-T2

i) Use of pH to elute antibody

The immediate effect of the addition of glycine/HCl to isoluminol was a 12% reduction in light output, with full recovery of light output by 5 minutes. Neutralisation with solid Tris resulted in an immediate reduction in light output by 30-80%, depending on how long the acid had been incubating with isoluminol, but delaying initiation of light output by 30 minutes following neutralisation resulted in recovery of light output to 100%.

Table 24 shows that less than 1% of labelled anti-HSV1 antibody bound to the control column, but 5.35% bound to the HSV1 column. However, nearly all the chemiluminescence was eluted by the Tween 20 washing, with only 0.15% being eluted by pH 2.7, suggesting that labelling of antibody was unsuccessful, immunoreactivity was destroyed, or the antibody was rendered lower affinity.

Eluant	Control antigen		HSV1 antigen	
	Control Ab*	Anti-HSV1 Ab*	Control Ab*	Anti-HSV1 Ab*
Unbound	99.00	99.27	99.53	94.65
Tween	0.94	0.60	0.40	5.20
pH 2.7	0.06	0.03	0.07	0.15
Seph 4B	0.00	0.00	0.00	0.00

* - percentage of total chemiluminescence added (mV/s)
Seph 4B - residual chemiluminescence in sepharose 4B

TABLE 24: Elution of Isoluminol labelled alpha-T2 from an antigen coated sepharose 4B column using pH.

ii) Assessment of IgG content of eluants by immunoblotting

pH elution did contain significant amounts of IgG from the alpha-T2 and HSV1 antigen incubation, with much less in the Tween 20 eluant and the majority of the antibody unbound in the washings. This suggested that labelling reduced the immunoreactivity of the antibody considerably.

C. Labelling and purification of a commercial monoclonal anti-HSV1 IgG

i) Sephadex chromatography and specific activity

Sephadex chromatography showed successful separation of the free and antibody-bound isoluminol into two distinct peaks (data not shown), and IgG immunoblotting confirmed the IgG content to be in the first peak, with a maximum concentration of 38 mg/l. The chemiluminescent activity of the first peak was confirmed, and the specific activity of the isoluminol labelled fractions was calculated at between 2.2 and 2.8.

ii) Affinity purification using pH and NaSCN

Table 25 shows that 52% of the chemiluminescent activity remained bound to the gel with no significant elution above control by pH.

Eluant	Control antigen Peak mV/s (%)	HSV1 antigen Peak mV/s (%)
Added	4100 (100.0)	4500 (100.0)
Unbound	3552 (86.6)	2112 (46.9)
pH 2.7	50 (1.2)	27 (0.6)
Seph 4B	478 (11.6)	2331 (51.8)
Total	4098 (99.4)	4470 (99.3)

Seph 4B - residual counts in sepharose 4B

TABLE 25: Elution of Isoluminol labelled monoclonal anti-HSV1 IgG from an antigen coated sepharose 4B column using pH.

Despite the inhibitory effect of NaSCN on chemiluminescence, Table 26 shows that 1 M still gave a value of 15% of added counts.

Eluant	Control antigen Peak mV/s (%)		HSV1 antigen Peak mV/s (%)	
Added	4150 (100.0)		3850 (100.0)	
Unbound	3950 (95.0)		1560 (40.5)	
10 ⁻⁶	100 (2.4)		180 (4.7)	
10 ⁻³	45 (1.1)		680 (17.7)	
1	0 (0.0)		600 (15.6)	
2	0 (0.0)		0 (0.0)	
3	0 (0.0)		0 (0.0)	
4	0 (0.0)		0 (0.0)	
Seph 4B	0 (0.0)		0 (0.0)	
Total	4095 (98.5)		3020 (78.4)	
Unacc	55 (1.5)		830 (21.6)	

Unacc - chemiluminescence unaccounted for
Seph 4B - residual counts in sepharose 4B

TABLE 26: Elution of Isoluminol labelled monoclonal anti-HSV1 IgG from an antigen coated sepharose 4B column using NaSCN.

The IgG immunoblots showed that the bulk of the IgG was in this 1 M NaSCN eluant.

Thus successful elution of labelled IgG was achieved.

D. Labelling and purification of human anti-HSV1 IgG

The IgG content of the purified serum was 28 g/l. Sephadex chromatography of the labelled antibody resulted in two peaks, the first of which showed IgG immunostaining only, and contained chemiluminescent fractions. The specific activity of the isoluminol labelled antibody was calculated at between 0.3 and 0.5.

Table 27 shows that pH eluted 4.8% of added chemiluminescence, with NaSCN eluting no counts above background, and 8.1% of chemiluminescence unaccounted for. IgG immunoblots, however showed that most of the IgG was in the 1 M to 4 M NaSCN eluants, with less in the pH and least in the tween washings.

Eluant	Control antigen Peak mV/s (%)		HSV1 antigen Peak mV/s (%)	
Added	16000	(100.0)	16000	(100.0)
Unbound	8540	(53.4)	6000	(37.5)
Tween	7140	(44.6)	7310	(45.7)
pH 2.7	135	(0.8)	764	(4.8)
NaSCN	0	(0.0)	0	(0.0)
Seph 4B	87	(0.5)	630	(3.9)
Total	15902	(99.3)	14704	(91.9)
Unacc	98	(0.7)	1396	(8.1)

Unacc - chemiluminescence unaccounted for
Seph 4B - residual counts in sepharose 4B

TABLE 27: Elution of Isoluminol labelled human anti-HSV1 IgG from an antigen coated sepharose 4B column using pH and NaSCN sequentially.

Thus the labelled IgG appeared to be of moderate to high affinity, and although there was no chemiluminescence in the NaSCN eluants, one could presume that the missing counts were 'hidden' in these fractions. At least 4.8% and up to 12% of the IgG was labelled and immunoreactive.

3. Discussion

Four antibodies were labelled and immunological and chemiluminescent functions assessed. Approximately 4-6% of labelled commercial antibody had immunological activity against HSV1 of high affinity; only 0.15% of labelled alpha-T2 antibody had immunoreactivity; labelled commercial monoclonal antibody contained at least 15%, and possibly 50% high affinity antibody; human labelled antibody showed between 4 and 12% moderate to high affinity antibody.

Chaotropic agents active enough to dissociate antigen-antibody complexes destroyed chemiluminescence and possibly immunoreactivity. NaSCN was the most effective chaotrope, but destroyed chemiluminescence at concentrations above 0.1 M. pH and NaCl were both less effective, but did not interfere with chemiluminescence if appropriate measures were taken with the former method. Affinity purification overall conferred no advantage over unpurified labelled antibody as a result. Labelling of the antibody also reduced the immunoreactivity considerably in some cases, presumably due to steric hindrance, partially explaining the low yields. Each antibody, therefore, had to be assessed fully, as prediction of outcome was difficult. The monoclonal antibody appeared to give the best yield of active labelled antibody of high affinity, and the highest specific activity of those 3 antibodies where this was assessed.

CHAPTER 8: CHOICE OF SOLID PHASE FOR THE ASSAY SYSTEM

In this chapter, 3 different solid phases were examined for suitability in a sandwich assay: Sepharose 4B, 5mm Polystyrene balls and polyvinyl microtitre plates (ELISA plates). The assays were performed initially using isoluminol labelled antibodies prepared in the previous sections, but as will be seen, and for reasons that will become apparent, luminol based chemiluminescence using HRP conjugates was reintroduced.

1. Materials and methods

A. Sepharose 4B as solid phase

i) Sandwich assay using alpha-T2 coupled sepharose 4B

IgG purified alpha-T2 antibody was coupled to sepharose 4B as in chapter 6. This choice of captor antibody was made because preliminary immunoblotting studies had shown this to contain the largest amount of high affinity anti-HSV1 antibody, next to the commercial monoclonal. 100 ul of the gel was incubated with 10 ul of 1:20, 1:220 and 1:2420 dilutions in PBS of HSV1 Kos pre-treated with 8M urea/1% triton X100 (U/T) (to expose epitopes for the detector antibody), or similarly treated measles antigen (Behring diagnostics, Hoescht UK Ltd., Hounslow, Middsx.) as control. Following incubation for 30 min at RT, gels were washed x 5 with PBS, then 10 ul of isoluminol labelled monoclonal anti-HSV1 IgG in 500ul PBS was incubated for 30 min at RT. The gels were washed x 5 in PBS, then 0.05% Tween 20 in PBS, the washings and the gels being counted using standard chemiluminescence reagents.

The lowest limit of detection (sensitivity) of the assay was taken as being when the HSV average counts of duplicate analyses failed to exceed the means of either the control or background counts by more than 2 SD of these values. The dilution immediately above this was taken as the limit.

Background cross reactivity between the rabbit and mouse antibodies and the mechanical effect of entrapment within the gel was established by incubating a range of amounts of the labelled monoclonal with 100 ul of gel for 40 min at RT, washing with PBS and Tween 20 as before and counting the gel.

ii) Effect of sepharose on light output

Mechanical interference in the light detection by the luminometer due to the sepharose gel was examined by mixing 100 ul gel with 100 ul of the isoluminol base at $1:10^{-6}$ or $1:10^{-5}$ dilutions in PBS or 10 ul of labelled monoclonal antibody, counting using standard reagents, and comparing with the chemiluminescence without the gel.

B. Polystyrene balls as solid phase

Several combinations of antibodies examined in previous sections were used, initially isoluminol labelled, but then luminol chemiluminescence was introduced, and a new set of antibodies was examined. Polystyrene balls, 5 mm diameter, were found to fit in the reaction cuvettes neatly, and the balls were later modified by coating with poly-phenyllysine and protein A to improve antibody binding.

i) Sandwich assay using isoluminol labelled antibodies

a) Coating balls with captor antibody

The method of ball coating was adapted from Wood et al [282]. All subsequent coating methods followed this, with minor modifications. 100 5mm polystyrene balls (Euro-matic Ltd, London, England) were mixed by intermittent agitation with 0.3 mg of purified alpha-T1 or 0.3 mg commercial monoclonal anti-HSV1 IgG in 12.5 ml 0.05 M carbonate buffer pH 9.5 at 4°C for 108 hr. The unbound sites were blocked by adding 1 ml of 20 mg/ml albumin, mixing for 24 hr at 4°C and adding a spatula tip of tris(hydroxymethyl)aminomethane buffer (tris; Sigma, Poole, Dorset) and mixing for a further 24 hr at 4°C. The excess was washed off with 0.15 M NaCl in a continuous flow, then distilled water, before drying in a stream of compressed air and storing in an airtight container at 4°C before use.

A rough indication of the success of protein uptake was made by incubating coated balls with goat anti-rabbit IgG HRP conjugate or rabbit anti-mouse IgG HRP conjugate (Atlantic antibodies, Winnersh, Berks.) 500 ul 1:2500 dilution in PBS, in 60 well microtitre plates (Abbott laboratories, North Chicago, Illinois) pre blocked with 1% gelatin (Sigma, Poole, Dorset)/1% albumin for 1 hr. The balls were washed with a Pentawash II automated ball washer (Abbott laboratories, North Chicago, Illinois), and 200 ul 10 mg ortho-phenylenediamine (OPD) (Sigma, Poole, Dorset) in 10 ml 0.1 M

PBS pH 6 activated with 10 ul neat H_2O_2 added to each well. The reaction was terminated by the addition of 200 ul 1 M H_2SO_4 . Balls were removed and the colour assessed on a visual scale (later studies were assessed spectrophotometrically).

b) Sandwich assay

Alpha-T1 or monoclonal antibody coated balls were incubated with 500 ul of 10-fold dilutions of U/T disrupted HSV Kos or measles antigen in microtitre wells for 1 hr at RT. After washing as above, 500 ul of 1:50 dilution of isoluminol labelled monoclonal or alpha-T2 antibody was added for 1 hr, the balls washed, transferred to reaction cuvettes, and chemiluminescence initiated with standard reagents.

ii) Sandwich assay using luminol based chemiluminescence

a) Re-evaluation of luminol chemiluminescence

Preliminary attempts at a sandwich assay were unsuccessful, mainly due to the high background and unpredictable kinetics of the luminol spike described in chapter 6. The effect of light and temperature had previously been examined, replacement with fresh reagents did not influence the reaction, but the effect of blocking agents, the mechanical influence of the ball and the use of 0.33 M NaOH in the H_2O_2 on chemiluminescence had not been examined.

Standard iodophenol enhanced luminol chemiluminescence was set up with a constant 1:4000 dilution goat anti-rabbit IgG HRP conjugate used as catalyst. Concentrations of albumin and gelatin from 0 - 0.05% were included, reactions with and without the ball were performed, and chemiluminescence initiated with and without NaOH in the H_2O_2 .

b) Sandwich assay

Macrotitre wells were blocked with 1% gelatin in PBS for 2 hr at RT, washed, then alpha-T1 coated balls incubated overnight at 4°C in 400 ul of 1:2000 - 1:128000 dilutions of U/T treated HSV Kos or measles in PBS. After washing, 450 ul of 1:500 commercial monoclonal in 0.1% gelatin in PBS was incubated for 1 hr at RT, washed with 0.1% tween 20/PBS then H₂O, 450 ul 1:500 affinity purified rabbit anti-mouse IgG HRP conjugate was incubated for 1 hr at RT, washed as before, then the balls read in the luminometer with 400 ul PBS, 20 ul 1:10 purified luminol, 20 ul 1:2 iodophenol and 50 ul H₂O₂ in H₂O, and counts integrated over 120 s.

Stability of the balls used in the assay was investigated by repeating the 1:5000 antigen incubation with the same batch of balls over a 2 week period, using fresh luminescence reagents each time.

iii) Poly-phenyllysine coating of balls

a) Ball coating

Poly-phenyllysine (PPL) covalently binds protein to polystyrene, and its use was hoped to improve the stability of the protein bound balls. The method of coating was taken from Wood et al. [282]. 2.5 mg PPL (Sigma, Poole, Dorset) was dissolved in 125 ml double distilled H₂O and enough to cover 100 balls used to coat with frequent agitation at RT overnight. 250 ul of 250 g/l glutaraldehyde in 15 ml H₂O was incubated for 40 min to activate the PPL, the balls washed, then protein coating with alpha-T1 took place as before (section B i) with gelatin substituted for albumin.

A rough assessment of protein uptake was also made as before (section B i), but using affinity purified goat anti-rabbit IgG HRP conjugate, and with the transference of 50 ul of the final colour product to a Dynatech ELISA plate reader (Dynatech laboratories Ltd., Billingshurst, Sussex) and reading at 425 nm..

b) Sandwich assay

The sandwich assay for HSV1 and measles was identical to section B ii, except antibody dilutions were 1:800 not 1:500, and the range of antigen dilutions was 1:4,000 - 1:32,000.

iv) Protein A as a surface binding agent

Protein A has been reported to enhance binding of IgG to surfaces and reduce inter-assay variability in similar systems [222]. The optimisation of coating with Protein A depends on the IgG coating concentration and has to be worked out empirically. Protein A coating would also improve the capture efficiency of the system by aligning the IgG molecule Fab portion 'upwards'. Because of the unsatisfactory results from the previous antibodies, a new range of antibodies was evaluated. A breakdown in the luminometer meant that many of the experiments relied upon conventional colorimetric analysis, but this later proved to be a more convenient screening tool, since large numbers of samples could be analysed at one time, rather than the sequential analysis required by the luminometer.

a) Optimisation of Protein A and IgG coating

Polystyrene balls were coated with Protein A (Seralab, Crawley Down, Sussex) by incubating with an appropriate amount in 50 ml 0.05 M carbonate buffer pH 9.5 at RT overnight to give a ball coating of 0.1 ug, 0.25 ug or 1.0 ug per ball. After washing, the balls were then coated as before (section B i) with human anti-HSV1 IgG to give a ball coating of 1 ul, 3 ul or 10 ul per ball (10ug, 30 ug or 100 ug), available protein A sites were saturated with normal human serum and unoccupied protein binding sites blocked as before but with gelatin in place of albumin.

IgG uptake was assessed by incubating the balls with goat anti-human IgG Fab specific, 150 ul of 1:1000 dilution in PBS for 1 hr at RT, then 150 ul rabbit anti-goat IgG HRP conjugate, 1:1000 in PBS, before visualisation with OPD as before and 100 ul of the solution transferred to the plate reader.

Effectiveness of antigen capture was examined by incubating the Protein A/IgG coated balls with 200 ul U/T treated HSV Kos or measles virus 1:100 in PBS for 3 hr at RT, washed as standard. The detector antibody was a rabbit R38/5 polyclonal IgG against nearly all HSV1 proteins (gifted by Dr. David Meredith from Leeds University), 150 ul at 1:1000 in PBS for 1 hr, and after washing 150 ul of 1:1000 in PBS goat anti-rabbit Fab fragment HRP conjugate (Sigma, Poole, Dorset) incubated for 1 hr at RT, then visualised with OPD and 100 ul read in a plate reader.

Reduction in background noise was attempted by incorporating 0.1% gelatin blocker in all the antibody stages and using ethylenediaminetetra-acetic acid disodium salt (EDTA) (Sigma, Poole, Dorset) at a variety of concentrations in the detector antibody incubation to reduce non-specific binding.

b) Sandwich assay

The sandwich assay was performed as above (section B iv a), using 0.1 ug/ball protein A coating, the 3 IgG coatings, 1:1000 - 1:50,000 U/T treated HSV Kos or measles, 0.1% gelatin in the incubation mixes, and 0.1 mM EDTA in the detector antibody. OPD staining was stopped at 10 min by 2 M HCl. The assay was repeated with the same batch of balls over a 6 week period to assess stability. In addition 3 new rabbit anti-HSV1 antibodies and a mouse monoclonal antibody were introduced into the assay system as both captors and detectors: rabbit 205/5 IgG polyclonal against non-envelope structural proteins and 220/3 IgG polyclonal against late protein VP 13/14 (ICP 22), both gifted by Dr. David Meredith from Leeds University), rabbit 50/4 IgG polyclonal against

alkaline nuclease and mouse monoclonal IgG Q2 against alkaline nuclease, both gifted by Dr. Ken Powell of Wellcome Research Laboratories. The appropriate normal serum was used to saturate the protein A sites in each batch.

v) Polyphenyl lysine and Protein A coating of balls

a) Optimisation and sandwich assay

Since PPL gave stability to protein coating, and protein A improved antibody coating, combining the two was assessed in the sandwich assay. Coating procedures took place as described above for both PPL (section B iii) and protein A (section B iv) using the 3 coating amounts for each, and all the antibodies used above as captor. A fixed 1:100 dilution U/T HSV Kos or measles virus was used to compare assays, with the appropriate species antibody as detector, and HRP Fab fragment conjugates as final antibody, goat anti-rabbit, sheep anti-mouse (Sigma, Poole, Dorset), or goat anti-human (Sigma, Poole, Dorset). OPD staining, neutralisation and reading was as before.

C. Microtitre (ELISA) plates as solid phase

i) Optimisation of reagents

The detector portion of the assay was first to be optimised. At the time of these experiments, only the early anti-HSV1 antibodies were available, but in the next chapter the full range were investigated.

96 well microtitre plates (Falcon 3912 microtest III, Becton Dickson Labware, Oxnard, California) were coated with 50 ul U/T treated HSV or measles antigen diluted in PBS from 1:100 to 1:10,000 for 2 hr at RT. Wells were then blocked with either 2% gelatin, 1% gelatin, 1% albumin, 1% casein, or an equimolar mixture of 1% gelatin and 1% albumin, or 1% gelatin and 1% casein in PBS for 1 hr at RT, the plates washed with 0.1% blocker in PBS with a final H₂O wash, then incubated with 50 ul 1:100 to 1:1000 dilutions in 0.1% blocker in PBS of commercial rabbit anti-HSV1, commercial mouse monoclonal or alpha-T1 for 1 hr at RT. After washing as before, a final incubation with 50 ul 1:500 to 1:5,000 in 0.1% blocker in PBS of non-affinity purified goat anti-rabbit IgG HRP conjugate or rabbit anti-mouse IgG HRP conjugate (Atlantic antibodies, Winnersh, Berks.), or affinity purified sheep anti-mouse IgG Fab fragment HRP conjugate (Sigma, Poole, Dorset) or goat anti-rabbit IgG Fab fragment HRP conjugate (Sigma, Poole, Dorset) for 1 hr at RT, washed again, then OPD added, neutralised after 20 min with 2 M HCl and read in the Dynatech plate reader.

ii) Sandwich assay comparison of colorimetric and chemiluminometric detection

50 ul of 1:50 dilution of alpha-T1 in 0.05 M carbonate buffer pH 9.6 was incubated in each well of a microtitre plate at 4°C overnight. Wells were then blocked with 150 ul 1% gelatin in PBS, washed with 0.1% gelatin in PBS then H₂O and duplicate wells incubated with serial dilutions of U/T HSV or measles in 1% gelatin in PBS for 1 hr at

RT. After washing 50 ul 1:50 in 1% gelatin in PBS mouse monoclonal was incubated for 1 hr at RT, washed, then 1:1000 rabbit anti-mouse IgG HRP in 1% gelatin in PBS incubated for 1 hr at RT, washed, then either colorimetric or chemiluminometric analysis standard reagents added. The wells for chemiluminescence were cut from the plate and placed into the reaction cuvettes, where the feasibility of using such wells had already been established without any reduction in light output from mechanical interference. Wells so examined were kept on ice until read.

The optical density and chemiluminescence counts were corrected for background $+2SD$, and plotted against the plaque forming units (PFU) content of the dilutions. The average of three duplicate wells for each dilution was calculated. If one value differed by more than 50% of the mean of the other two it was discarded.

In the subsequent text, figures and tables, where PFU is given, it is to be read as equivalent to 10^{-2} infected cell products (ICP) i.e. $1 \text{ ICP} = 10^2 \text{ PFU}$.

2. Results

A. Sepharose 4B as solid phase

i) Sandwich assay using alpha-T2 coupled sepharose 4B

The gel reduced light detection by between 25-47%, presumably through quenching, but this effect would act equally on control and test specimens. Cross-reactivity between the labelled mouse monoclonal and the rabbit antisera coated gel was less than 0.5% for all concentrations of labelled antibody.

Figure 13 shows that sensitivity of the sandwich assay was poor at only 1:220 dilution of HSV Kos (5×10^5 PFU), with low signal to noise and high background.

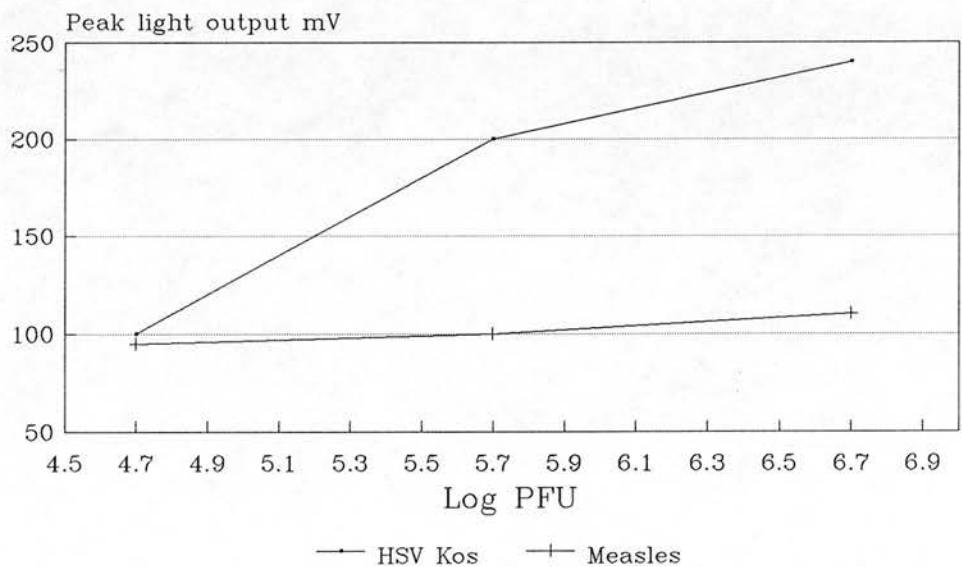


Figure 13 : Sensitivity of sepharose 4B
Isoluminol based antigen capture assay

B. Polystyrene balls as solid phase

i) Sandwich assay using isoluminol labelled antibodies

Significant antibody coating of the balls was confirmed by OPD staining for both alpha-T1 and mouse monoclonal antibodies.

The sandwich assay showed very poor sensitivity with only a 1:100 dilution of HSV Kos (5×10^7 PFU) being detected by alpha-T1 captor, and no significant detection when the monoclonal antibody was the captor. However background binding was low in this system.

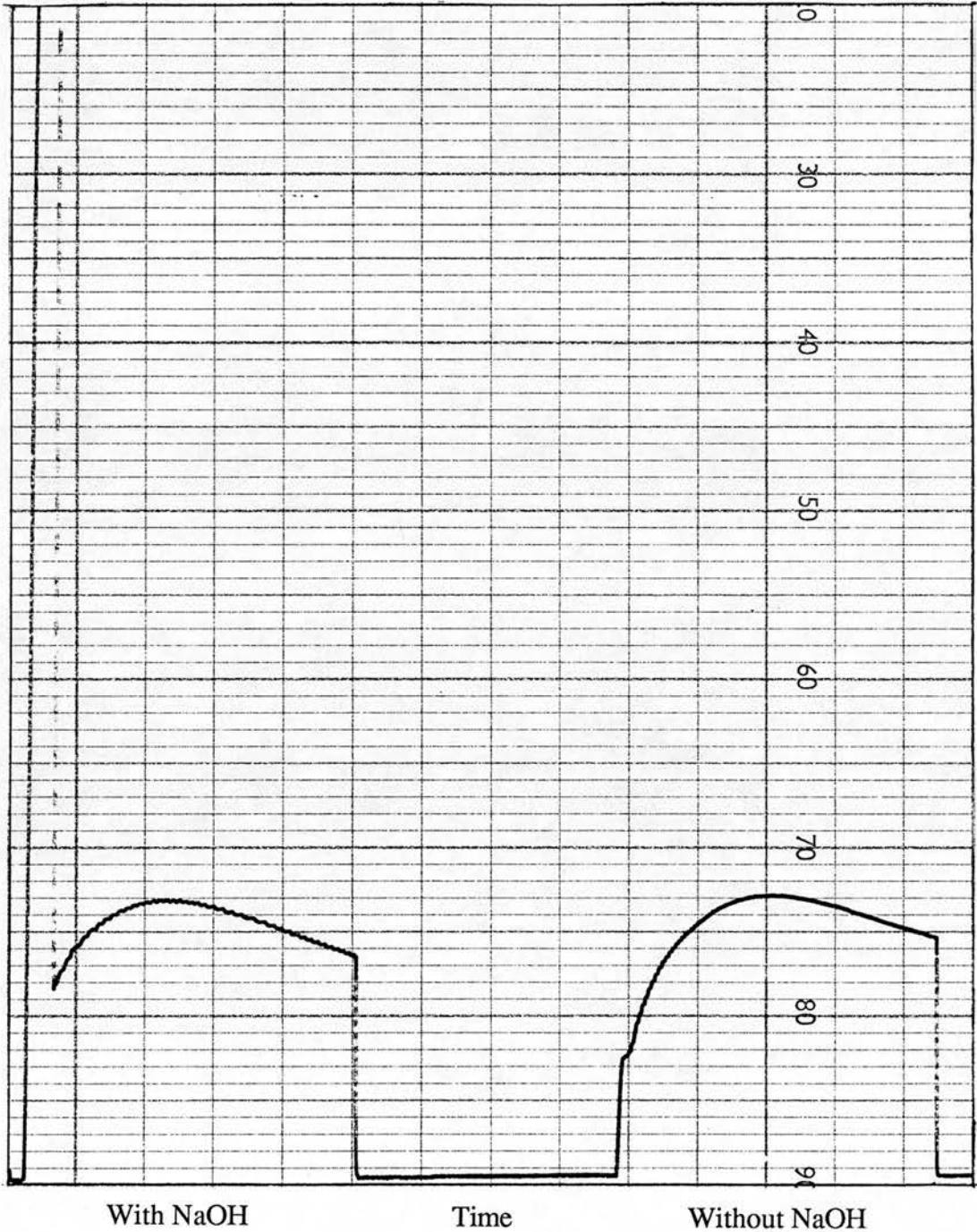
ii) Sandwich assay using luminol based chemiluminescence

a) Re-evaluation of luminol chemiluminescence

Albumin inhibited chemiluminescence by >50%, even at 0.005% concentrations, whereas gelatin resulted in only 7% inhibition at the same concentrations. The mechanical interference by the ball resulted in between 30-40% reduction in light detection.

Figure 14 shows, however that the major cause of the poor performance of luminol was the presence of NaOH in the H_2O_2 . Removal resulted in abolition of the initial spike, and enhancement with iodophenol of >1000 fold, as described in the literature.

Light output mV/s



| 20s

Figure 14 : Effect of NaOH on the kinetics of p-Iodophenol enhanced Luminol chemiluminescence

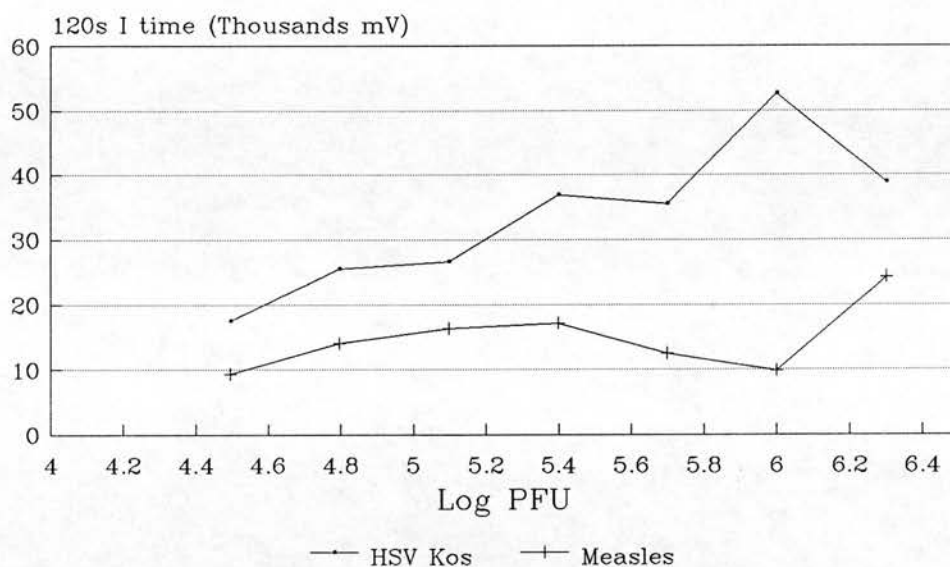


Figure 15 : Sensitivity of polystyrene ball Luminol based antigen capture assay

b) Sandwich assay

Figure 15 shows that the assay sensitivity was improved considerably, with a 1:128000 dilution of HSV Kos detected (3×10^4 PFU). The cross reactivity with measles was low, and only the maximum concentration of measles exceeded the any of the HSV Kos results. The use of affinity purified HRP conjugates reduced the background levels considerably.

The assay was, however, unstable, with the complete loss of sensitivity by 2 weeks, and the HSV:measles counts reduced to 1.1 for the 1:5000 antigen dilution.

iii) Poly-phenyllysine coated balls sandwich assay

PPL improved the coating with IgG by 25%, but the sandwich assay was a failure with only a 1:4000 dilution of HSV Kos detected (10^6 PFU), mainly because of a huge increase in background counts. The assay results were repeatable over a 2 week period, so although PPL did improve stability, it reduced sensitivity 30-fold.

iv) Protein A coated balls sandwich assay

The optimum protein A coating was 0.1 ug per ball, and the optimum IgG coating for maximal IgG uptake was 1 ul (10 ug). The optimum HSV antigen capture at 1:100 dilution of antigen was achieved by the 0.1 ug protein A/10 ug IgG coated ball.

Incorporation of 0.1% gelatin blocker into all incubations, and the use of 0.1 mM EDTA in the detector antibody incubation, reduced background by 58% and 10% respectively for the optimum ball coating.

The best assay combination, which was unexpectedly the 30 ug/ball human IgG coating, produced a sensitivity of only 1:10,000 HSV Kos (4×10^5 PFU). All other protein A and antibody coating concentrations, for all the other appropriate combinations of captor and detector antibodies used failed to give a sensitivity above 1:1000 HSV Kos (4×10^6 PFU). Repeat assays with the best assay over 6 weeks saw a complete loss of sensitivity.

v) Poly-phenyllysine and protein A coated balls sandwich assay

Only 0.25 ug protein A with 10 ug human IgG or 30 ug rabbit 205/5 were successful in capturing HSV Kos at 1:100 dilution. All other combinations gave negative results. The human IgG gave a 1:1000 sensitivity finally (4×10^6 PFU), but the 205/5 could not improve on 1:100. The background counts were again all high accounting for much of this poor sensitivity.

C. Microtitre (ELISA) plates as solid phase

i) Optimisation of reagents

1% gelatin blocking gave the lowest background counts of the 6 blocking mixtures. The best signal to noise for detector antibodies was obtained with 1:500 dilutions of the alpha-T1 and monoclonal, and 1:1000 dilution of commercial antibody. The non-affinity purified anti-mouse HRP conjugate gave better results than the affinity purified Fab fragment HRP conjugate, and 1:500 dilution was optimum. The affinity purified Fab fragment anti-rabbit HRP conjugate was better than the non purified HRP conjugate, and 1:1000 dilution was optimum. Overall the best combination for signal:noise was the alpha-T1 detector and affinity purified Fab fragment HRP conjugate which also had the best sensitivity of 1:10,000 antigen bound to the plate. However the monoclonal antibody gave the lowest and cleanest background signal. In the sandwich assay, the alpha-T1 was thus used as the captor, and the monoclonal as the detector.

ii) Sandwich assay comparison of colorimetric and chemiluminometric detection

Figure 16 shows that, using alpha-T1 as captor and commercial monoclonal as detector, both methods were relatively insensitive, with the colorimetric particularly giving a poor slope, due in part to high background counts. Chemiluminescence achieved a sensitivity of 1:20,000 dilution of HSV Kos, or 2.5×10^4 PFU, and colorimetry 1:10,000 dilution, or 5×10^4 PFU, although the curves were irregular and somewhat inconsistent.

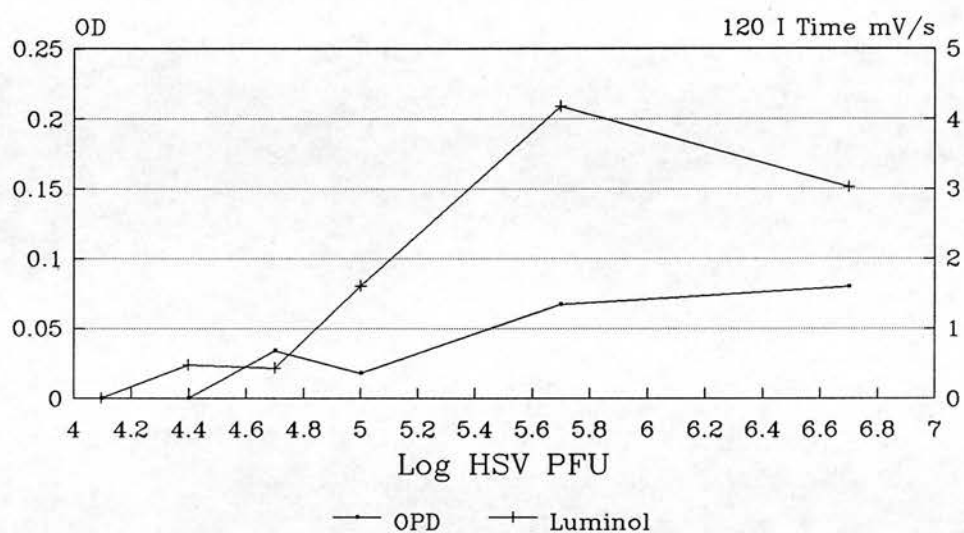


Figure 16 : Comparison of colorimetric and chemiluminometric detection of HSV using alpha-T1 captor, Q2 detector

3. Discussion

Sepharose was difficult to handle, required multiple wash-centrifugation steps, and so was time consuming and only limited numbers of assays could be performed concurrently. The coupling of antibody was efficient, but again was time consuming, although the stability of the gel was good. The assay was not sensitive enough using isoluminol labelled antisera, although it must be pointed out that luminol luminescence was not attempted with sepharose and the range of antibodies available at the time of these experiments was limited.

Polystyrene balls were much easier to handle, especially with the automated ballwasher, but sample volumes would have had to have been adjusted to a minimum of 200 ul and preferably 400 ul for effective ball coverage, thus introducing a further dilution step. Despite the promise offered by coating the balls with protein A and PPL the assay results were much worse than untreated balls, although the instability of the latter made what initially looked to be the most promising assay unsuitable. The failure of protein A lay partly in the difficulty in blocking all the unbound sites after the initial captor antibody binding, since steric hindrance occurred if the coating concentration was too high, and detector antibody binding took place if the concentration was too low. The breakdown of the luminometer meant that strictly accurate comparisons could not be made using protein A and PPL with other assays based on chemiluminescence, but as was seen with the ELISA plate based assay, the colorimetric analysis had similar sensitivity. The failure of PPL to improve the assay was unexpected, since it did improve stability of the protein binding. Mechanical interference had an effect greater than that seen with sepharose, and thus reduced sensitivity.

Luminol chemiluminescence was found to have been undervalued in the previous chapters because of the adverse effect of NaOH on the light kinetics. It was likely that the NaOH was generating free O^- and so initiating the spike which was independent of the HRP catalysed O^- production. Enhancement with iodophenol was returned to the levels expected, and resulted in a 1000-fold improvement in sensitivity of the assay.

Microtitre plates were easy to handle, coped with multiple assays in parallel, and the wells did not interfere with light detection when individual wells were cut out and placed in the reaction cuvettes. Although the assessment of antibodies was limited to only a few, the resulting sensitivity was equal to that seen with the polystyrene balls, and chemiluminescence achieved slightly better sensitivity than colorimetric detection.

Table 28 summarises the sensitivities achieved by the different assay systems. Overall, the microtitre based system offered the greatest promise, so the further development of the assay was based on this as a solid phase.

Solid Phase	Detector system	Sensitivity (PFU)
Sepharose 4B gel	Isoluminol	5×10^5
Polystyrene ball	Isoluminol	5×10^7
	Luminol	3×10^4
+PPL	OPD	1×10^6
+P-A	OPD	4×10^5
+PPL, P-A	OPD	4×10^6
Microtitre plate	OPD	5×10^4
	Luminol	2.5×10^4

P-A - protein A

TABLE 28: Comparison of HSV1 assay sensitivities using different solid phases and detector systems

CHAPTER 9: OPTIMISATION OF THE MICROTITRE PLATE BASED CAPTURE ASSAY

This chapter describes further the sandwich assay based on the microtitre plate using antibodies not previously assessed using this solid phase. The assay conditions were optimised, and a detachable well suitable for chemiluminometry was introduced. The comparison between colorimetric and chemiluminometric detection was made after initial work based on colorimetry, because of the ease of screening using this method.

1. Materials and methods

A. Optimum captor-detector combination

i) Monoclonal Q anti-alkaline nuclease as captor

Mouse monoclonal Q2 (Q2) against HSV alkaline nuclease was initially used in conjunction with 50/4 rabbit polyclonal (50/4) against the same alkaline nuclease. As mentioned in chapter 4 of the introduction, alkaline nuclease is one of the most abundant polypeptides produced during active replication of HSV, and results from transcription by B-genes in the second phase of viral replication. When 50/4 was used as the captor, the results were poor, so the assay was reversed, and in addition, the human and rabbit polyclonal antisera introduced to compare.

The microtitre plate assay conditions were set as finally achieved in chapter 8, with the following modifications. The coating concentration of Q2 was optimised at 1:1000 dilution. The incubation of Q2 was overnight at 4°C, first blocking was for 3 hr at RT. HSV Kos was treated by either sonication or 0.1% tween 20 (as preliminary experiments showed that U/T destroyed immunoreactivity of the relevant epitopes), and measles virus and vero cells (cells uninfected by HSV Kos, gifted by Dr. Robert Honess) were used as

controls and similarly treated. Antigens were diluted 10-fold and incubation was for 2 hr at 45°C, and detector antibodies 50/4 and R38/5 were optimised at a dilution of 1:400, human anti-HSV1 IgG at 1:100. The HRP conjugates were affinity purified Fab fragment antibodies, and the dilutions optimised at 1:1000. The OPD reaction was terminated at 30 min.

All assays were done in triplicate, the average readings taken, and if one value differed by more than 50% from the mean of the other two, it was discarded. The resulting optical densities were plotted as the ratio of the HSV:control value. The control values for measles virus were identical to those for vero cells, so in all later experiments vero cells alone were used as controls, with the advantage that the relevant dilutions could be exactly matched to HSV Kos. The blank value was the average of 10 readings, and cutoff was set at $\pm 2SD$. Ratios below 1.25 were judged to be negative.

ii) R38/5, 205/5, 220/3 and human anti-HSV1 IgG as captors

Methods were as above, with antigen 0.1% tween 20 treated, and with antibody dilutions modified to those detailed by experimentation:

R38/5 when captor was used at 1:500 dilution, detectors Q2 1:1000 and human IgG at 1:500;

205/5 when captor used at 1:500 dilution, detector human IgG at 1:1000;

220/3 when captor used at 1:1000 dilution, detector human IgG at 1:1000;

Human IgG when captor used at 1:1000 dilution, detectors 50/4, 220/3 at 1:400, R38/5, 205/5 at 1:1000 and Q2 at 1:2000.

HRP conjugates were all affinity purified Fab fragment antibodies, except when Q2 was a detector, when the non purified conjugate was used.

B. Use of Immulon detachable wells

Immulon I removawell strips (Dynatech Laboratories, Billingshurst, Sussex) are detachable microwells with a precoating of a polymer for covalent binding of proteins. The convenience of easy handling and correct size for the reaction cuvettes made them the ideal choice for the chemiluminometric assay.

i) Comparison of Immulon wells with microtitre plates

The assay was run using Immulon wells and microtitre plates in parallel using the materials and methods optimised in section A, but as the Immulon wells were larger volume, 350 μ l blocker was needed. Test: control ratios were plotted as before.

ii) Comparison of colorimetric and chemiluminometric detection

The assay was run as optimised on microtitre plates and duplicated on Immulon wells. One set of the Immulon wells to be used in the luminometer were kept on ice after the HRP incubation until read using standard iodophenol enhanced chemiluminescence. The other set and the microtitre plate were conventionally assayed with OPD. Signal:noise ratios were then plotted.

2. Results

A. Optimum captor-detector combination

i) Monoclonal Q2 as captor

Figure 17 shows that Q2 captor and 50/4 detector had the best sensitivity at $1:10^6$ dilution of 0.1% tween treated HSV Kos (20 PFU). Without Tween 20 the assay was less sensitive, by a factor of 10, presumably because Tween 20 was required to disrupt the intact cells and virions. Figure 18 shows the other detector antibodies were not as sensitive, with only 205/5 and R38/5 just achieving $1:10^5$ detection (200 PFU). Background counts were not as low as in preliminary experiments, because of the altered conditions of incubation to achieve higher sensitivity.

ii) Other antibodies as captors

Figures 19 to 21 show the results from the other antibody combinations. None achieved the sensitivity of Q2 and 50/4 combination, with the best being R38/5 captor and human IgG just significant at $1:10^5$ (200 PFU).

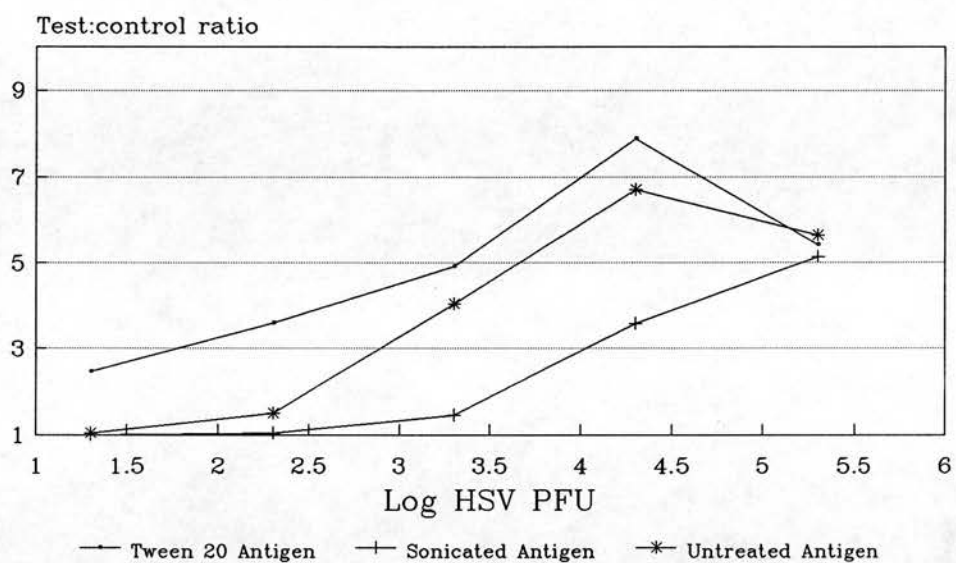


Figure 17 : HSV colorimetric sandwich assay with Q2 captor, 50/4 detector.

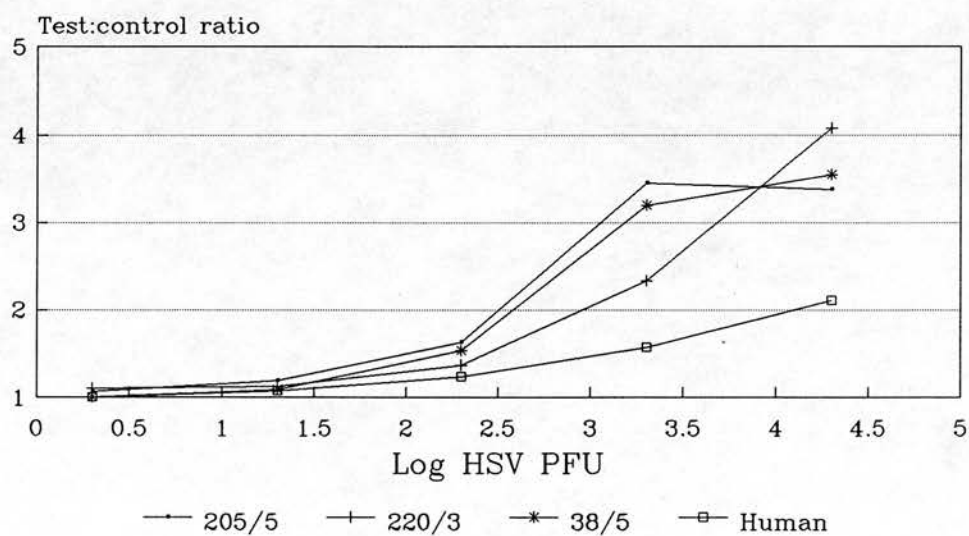


Figure 18 : HSV colorimetric sandwich assay using Q2 captor, and detectors 205/5, 220/3, 38/5 and human antisera.

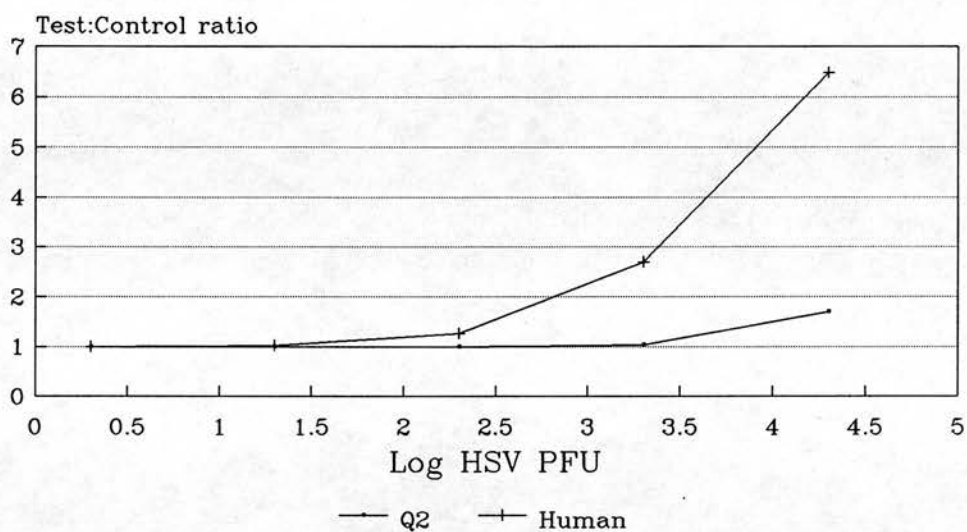


Figure 19 : HSV colorimetric sandwich assay with R38/5 captor, Q2 and Human antisera detector

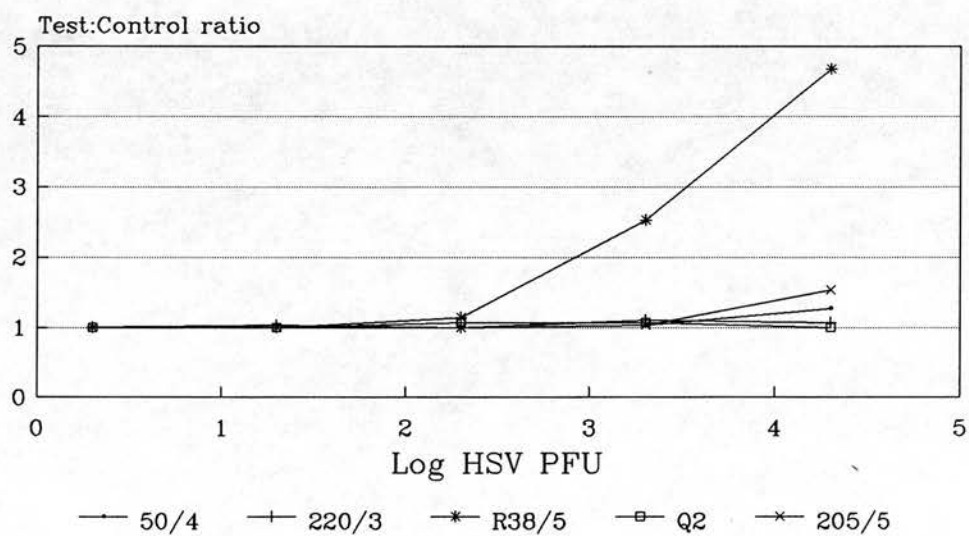


Figure 20 : HSV colorimetric sandwich assay with human antiserum captor, 50/4 220/3, R38/5, Q2, and 205/5 detector

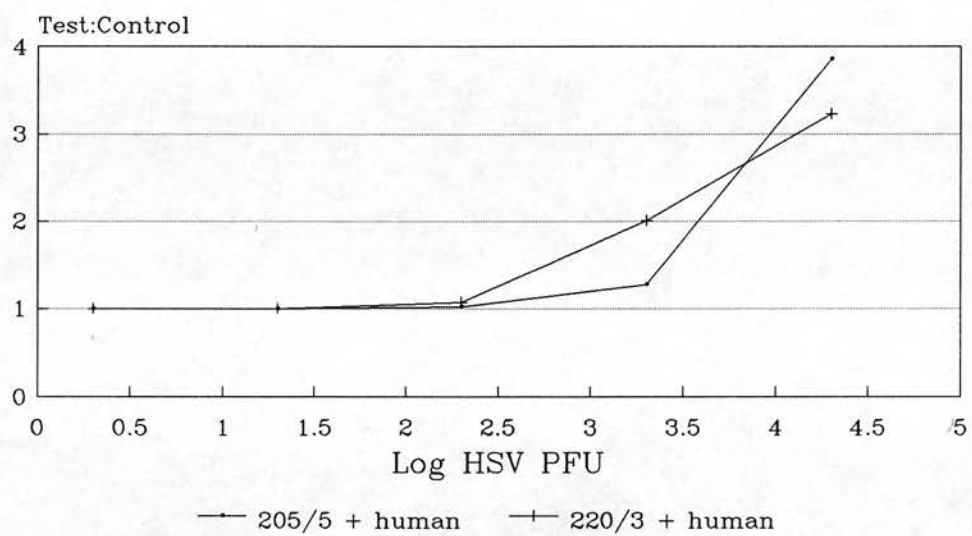


Figure 21 : HSV colorimetric sandwich assay with 205/5 or 220/3 captors, human antiserum detector

B. Use of Immulon detachable wells

i) Comparison of Immulon wells with microtitre plates

Figure 22 shows that using the colorimetric assay the Immulon wells performed as well as the microtitre plates in sensitivity, with an almost identical curve profile and a lower background.

ii) Comparison of colorimetric and chemiluminometric detection

Figure 23 shows that chemiluminescence was no more sensitive than OPD, although at higher HSV Kos concentrations, the signal:noise ratio was much better using chemiluminescence.

The latter experiments and figures show an apparent loss of sensitivity in the assay, as a result of a flattening of the curve. This was subsequently found to be due to destruction of the alkaline nuclease in the calibrant HSV Kos from prolonged incubation in Tween 20, and when incubation was limited to overnight sensitivity was found to be restored.

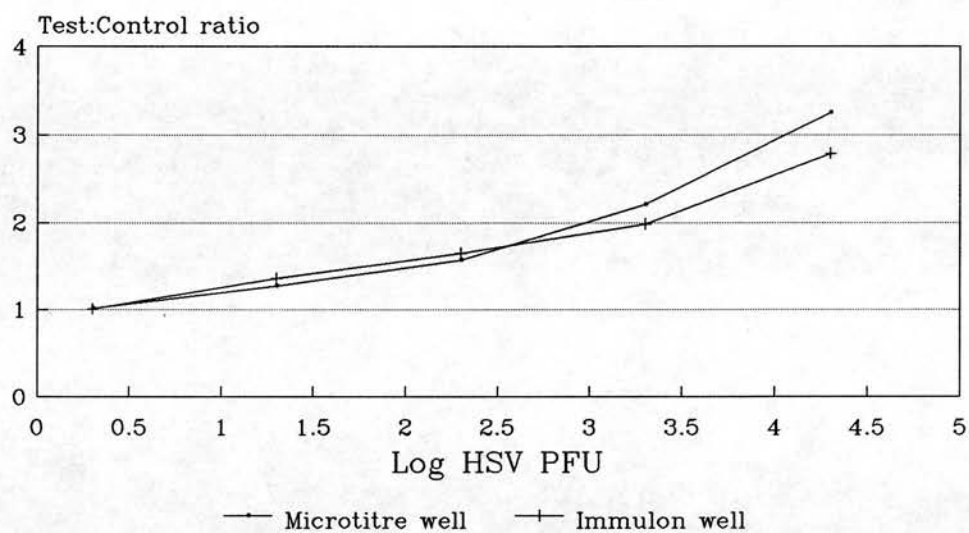


Figure 22 : Comparison of colorimetric assay using microtitre plates and Immulon wells

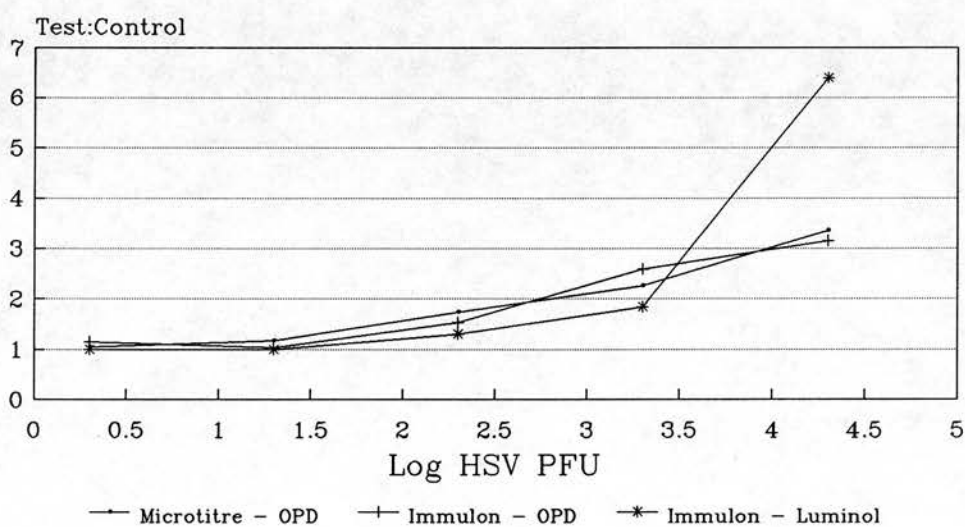


Figure 23 : Comparison of colorimetric and chemiluminometric detection systems in HSV assay

3. Discussion

Q2 mouse monoclonal anti-alkaline nuclease and rabbit polyclonal anti-alkaline nuclease were the most sensitive combination, able to detect 20 PFU of HSV. This paradoxical superiority of a monoclonal capture system probably reflected the 'cleanness' of the antibody, and because the polyclonal had other epitopes to link with there was no interference with binding which obviously took place when the assay was reversed. No other antibody combination achieved this sensitivity. Prolonged incubation beyond 2 hr for antigen did not result in better sensitivity, but raising the incubation temperature did, even though background was increased as a result. Measles virus in this assay gave the same counts as the vero cell control, which were at or near the background, again reflecting the specificity of the monoclonal, and the use of affinity purified Fab fragment HRP conjugate. For the remainder of the experiments vero cell controls alone were used. Gelatin blocker was, fortuitously for chemiluminescence, the most efficient.

The performance of chemiluminescence was a major disappointment. The sensitivity in the assay was no better than colorimetric analysis, and the mechanics of luminometry far more time consuming and prone to external influence. So saying, the colorimetric assay was extremely sensitive and it would have been more than one could reasonably expect to improve upon it. Chemiluminescence was thus abandoned in favour of colorimetric detection.

The requirement for Tween 20 pre-treatment of antigen to access the alkaline nuclease would hopefully not be required in the working assay, as necrotic tissue would release large amounts of non cellular or virion associated protein. This assumption could only be tested by running the assay on human specimens, and its validity would be apparent if the results were negative.

CHAPTER 10: DISSOCIATION OF ANTIGEN-ANTIBODY IMMUNE COMPLEXES

In chapter 2 it was proposed that part of the reason for the difficulty in the detection of antigen in CSF and the failure of culture of viruses from CSF was the result of immune complex formation. A variety of ways of dissociating antigen from antibody have already been described in Chapters 6 and 7 of this materials, methods and results section, and acid elution was found to be effective, without necessarily destroying antibody. Whether antigenicity of the viral epitopes was retained was not evaluated.

Acid dissociation of immune complexes in serum for HIV1 antigen detection has been incorporated into standard assays [266], so the next experiments were designed to see if this could be done with HSV antigen-antibody complexes created in vitro. Initially, acid elution was performed as described, then subsequently modified.

1. Materials and methods

A. Use of HCl to dissociate antibody from bound antigen

By coating microtitre plates with HSV, immune complexes could be generated by the addition of anti-HSV1 antibodies. The effect of acid on dissociation, and the retention of the antigenicity of the bound HSV was examined in this section. First, the effect of acid on a protein coated plate was examined.

i) Resistance of bound protein to acid removal

Duplicate wells of microtitre plates were coated with 10-fold dilutions of 50 µl goat anti-human IgG (Fc specific) (Atlantic antibodies, Warriner, Berks.) in 0.1% gelatin in PBS for 1 hr at RT. 50 µl of 0.5 M HCl was applied to one set of wells for 90 min at RT, then washed; one set had no acid applied and one set no coating antibody or acid. 50 µl 1:1000 dilution in 0.1% gelatin in PBS of rabbit anti-goat IgG HRP conjugate was added for 30 min to all wells, washed, then stained with OPD as standard and read in the plate reader.

ii) Dissociation of antibody from bound antigen

50 µl of 1:50 dilution HSV Kos in 0.05 M carbonate buffer pH 9.6 was used to coat overnight each well of a microtitre plate. Wells were then blocked with 1% gelatin in PBS, and after washing 50 µl of 10-fold dilutions of human anti-HSV1 IgG in 0.1% gelatin in PBS were incubated in duplicate wells for 90 min at RT. After washing, 50 µl 0.5 M HCl was added for 90 min to one set of wells, the other set left in 0.1% gelatin, then both washed. 50 µl of 1:1000 rabbit anti-human IgG HRP conjugate (Dako, High Wycombe, Bucks.) in 0.1% gelatin in PBS was added to each well and incubated for 1 hr at RT, then the wells were washed, stained with OPD as standard and read in a plate reader.

iii) Retention of antigenicity after acid treatment

Methods were as for section ii), with the exception that the human anti-HSV IgG was reapplied at the same dilution after acid treatment, and 0.1 M HCl was compared with 0.5 M. To ensure that the antibody was removed, not inactivated, 10-fold dilutions of HSV Kos were applied to wells, and a single 1:1000 dilution of human anti-HSV1 IgG added, to ensure that at some stage antibody was in excess. 0.1 M HCl only was used.

B. Sandwich assay to detect antigen dissociated from immune complexes

Arbitrary amounts of antigen and antibody were incubated together to form complexes.

With antigen in excess, little change would be seen after acid dissociation, but with antibody in excess, despite competition with antibody bound to the plate for free antigen, any change after acid treatment would indicate successful disruption of antigenically active epitopes.

i) Assay of dissociated antigen

200 ul of 1:1000 or 1:10,000 dilutions of HSV Kos in 0.05 M PBS were incubated with 200 ul of human anti-HSV1 IgG at 1:100, 1:1000 and 1:10,000 dilutions in PBS for 1 hr at RT. 100 ul of the mixture was then treated with 25 ul 0.5 M HCl (making final concentration HCl 0.1 M) at RT for 90 min, then neutralised with exactly 25 ul of 0.5 M NaOH and made up to 200 ul with H₂O, so the final dilution was 1:2. Controls without antibody added or acid incubation were also diluted 1:2. Samples were added to the standard Q2 captor, 50/4 detector assay system as soon as possible after neutralisation to prevent undue reformation of immune complexes, then the assay run as standard.

2. Results

A. Use of HCl to dissociate antibody from bound antigen

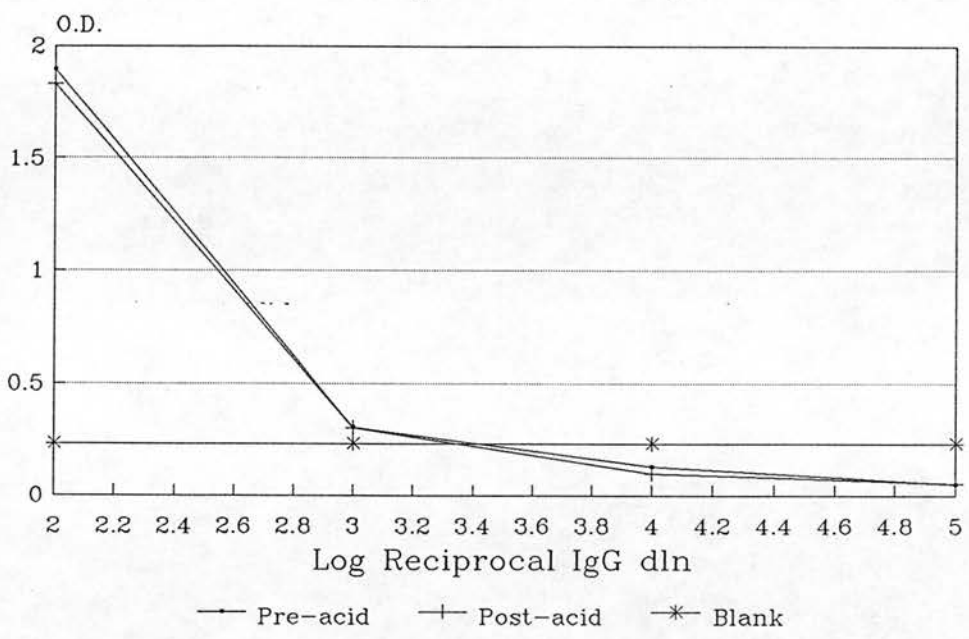


Figure 24 : Effect of 0.5 M HCl on IgG bound to microtitre well

Figure 24 shows that acid treatment made no difference to the to the amount of IgG coating the well, with identical calibration curves, and since the higher antibody dilutions gave lower O.D. readings than the controls with only the HRP conjugate added, no protein was being stripped from the wells.

Figure 25 shows that acid treatment successfully reduced the O.D. readings for each antibody dilution added to the fixed dilution of bound antigen, suggesting that the antibody was either eluted, the Fc portion rendered unrecognisable without dissociation, or the antigen rendered unrecognisable. The re-application of the first antibody restored the O.D. to pre-acid treatment levels, indicating that the HSV antigen was not destroyed or inactivated, and that the antibody was not inactivated, but eluted. However this experiment did not take into account the possibility that antigen may have been in such excess that second application of antibody was merely binding to previously unoccupied sites. Figure 26 shows that using a range of antigen concentrations and a fixed antibody concentration, even at great antibody excess, acid dissociated immune complexes, then

re-application of antibody resulted in a return to the previous O.D. levels, and not beyond, indicating that the antibody was eluted, not inactivated, and antigenicity was preserved.

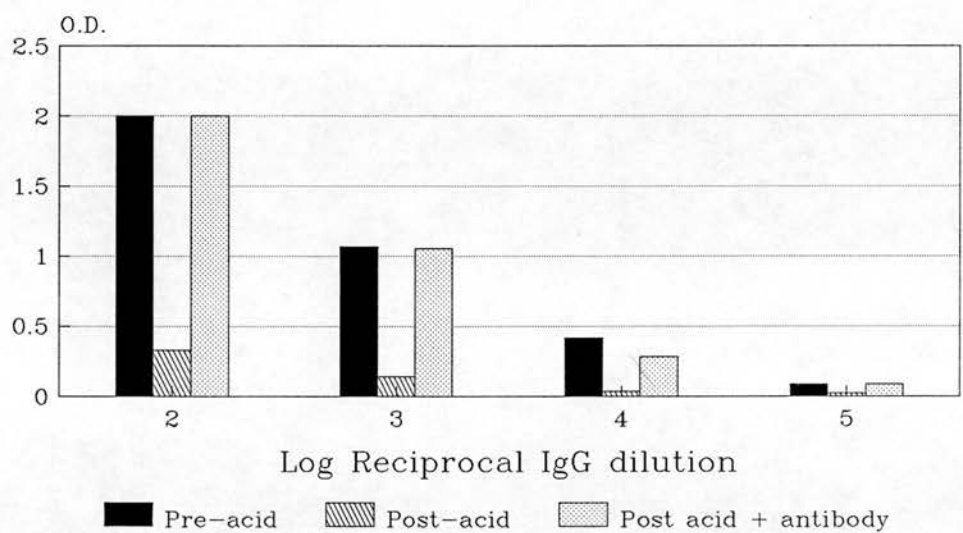


Figure 25 : Dissociation of Ab from Ag bound to microtitre well using 0.5 M HCl and retention of antigenicity

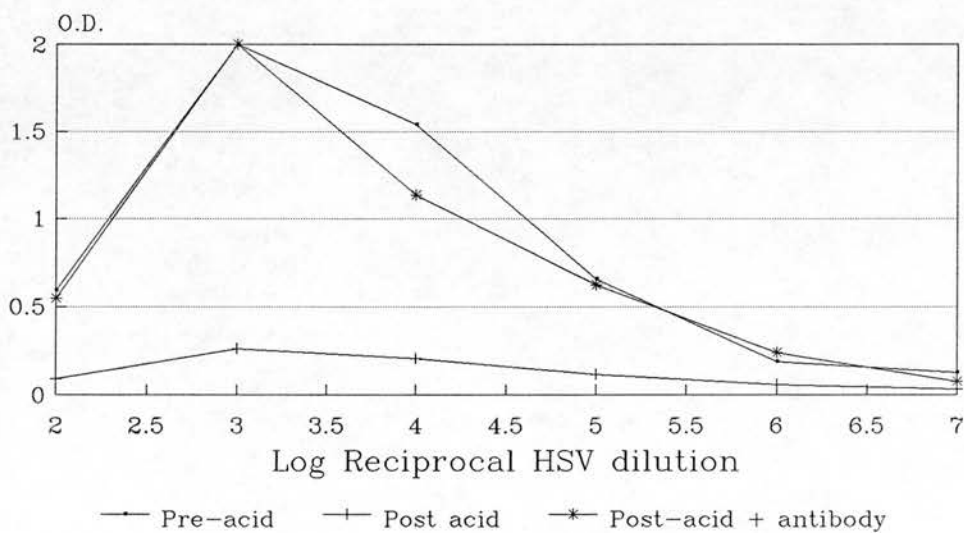


Figure 26 : Dissociation of Ab from Ag dilutions bound to microtitre wells with 0.1 M HCl and retention of antigenicity

B. Sandwich assay of dissociated antigen

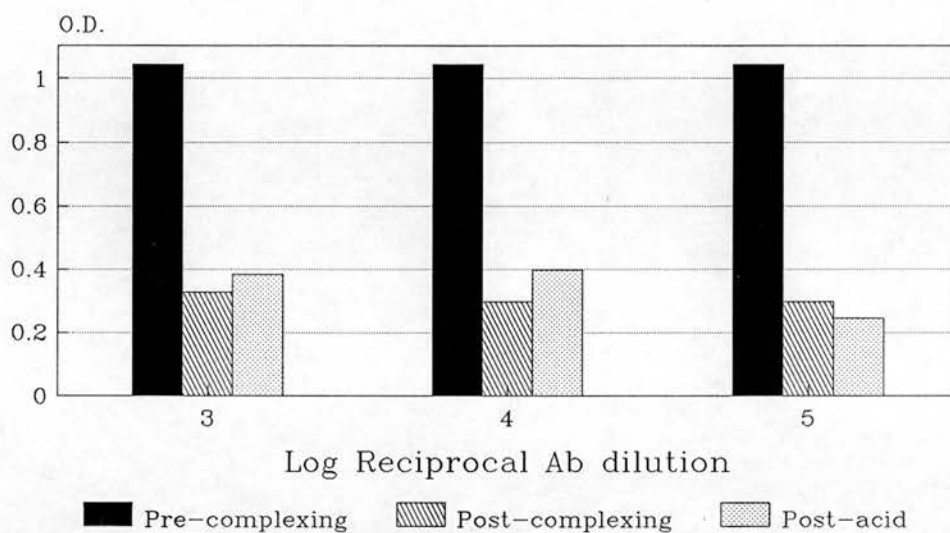


Figure 27 : Sandwich assay of 1:1000 HSV complexed with antibody dilutions before and after dissociation with 0.1 M HCl

Figure 27 shows that there was recovery of antigen from immune complexing between the 1:1000 dilution of antigen and 1:1000 and 1:10,000 dilutions of antibody. Recovery was low, at best being 14% of the bound antigen.

3. Discussion

The use of acid to dissociate immune complexed HSV Kos with human anti-HSV1 IgG was shown to be possible, and to leave the epitopes recognisable by the same antisera. The use of 0.1 M HCl was as effective as 0.5 M HCl. The low yield of recovered antigen may still indicate irreversible damage to the viral epitopes, or the failure to separate antibody of high affinity from the antigen. In any case having established the principle, the next stage was to assay the human CSF samples.

CHAPTER 11: CSF ANTIGEN ASSAY FROM PATIENTS WITH HSVE

1. Materials and methods

A. Patients

In the standard assay the following patients were studied:

12 CSF samples from 9 patients with definite HSVE were obtained from in patients at the National Hospitals for Nervous Diseases, and direct referrals from local hospitals in the region. Although initially promised samples held in a specimen library at the virology department of the University of Manchester, permission to use them was refused at a later date. Clinical and laboratory details of these patients is given in appendix 1. Only one patient had isolation of virus from CSF, none underwent brain biopsy, but the remaining 8 fulfilled the immunological criteria by having paired sera and CSF showing rising titres and local synthesis of specific IgG, although some had the diagnoses based on samples not available for this study.

A total of 35 control patients were used, all in-patients at the National Hospitals for Nervous Diseases, and consisted of 10 patients with infections of the nervous system, including one HSV myelitis, one tuberculous meningitis (TBM) with reactivation of HSV and one post HSV Guillain-Barre syndrome (GBS), 6 patients with demyelinating disease, all clinically definite multiple sclerosis (MS), 2 with inflammatory CNS disorders, 7 with a variety of neurological diseases, such as dementia, cerebral tumours or degenerations, and 5 patients with non-CNS disorders, such as headache or prolapsed intervertebral discs. Finally a group of 5 patients with suspected HSV infections, 4 encephalitis and one myeloradiculopathy, were included, with clinical features consistent with the diagnosis, but without sufficient laboratory supportive evidence.

For the HSV1 antigen index an overlapping population of patients was used, consisting of 4 CSF samples from 3 of the definite HSVE patients above, and 37 control patients, again all in-patients at the National Hospitals for Nervous Diseases. The control group consisted of 19 patients with CNS infectious disorders, 2 patients with MS, 4 patients with inflammatory diseases of the CNS, 4 patients with other neurological diseases and 8 patients with suspected HSVE. Of these 37 patients, 7 with infections, both cases of MS, 1 inflammatory, all 4 other neurological diseases and 3 suspected HSVE had also been examined by the standard ELISA assay below.

B. Sandwich capture assay

i) The standard assay

The assay was set up as optimised in chapter 9. Each plate incorporated a standard HSV Kos and vero cell calibration curve. Once a positive CSF had been identified with sufficient sample volume, all subsequent plates contained this as a positive control. CSF samples from the patients with HSVE were treated identically to the CSF samples from control patients, which were all coded and then assayed blind.

50 ul of CSF was incubated with 25 ul 0.3 M HCl (final concentration 0.1 M HCl), for 90 min at RT, then neutralised with 25 ul 0.3 M NaOH and immediately added to the well. 100 ul of a 1:2 dilution in 0.15 M NaCl of untreated CSF was placed in the paired well. The assay then proceeded as standard.

Assays were run several times, when sample volumes permitted, to ensure reproducibility of the results, using a variety of control samples. Results were expressed on the basis of comparison with the calibration curve corrected for background, and reading the background corrected values for the samples against this curve. Test readings below 2 SD of the mean above background were considered negative. Average corrected values for duplicate samples were calculated.

ii) HSV1 antigen index

In chapter 2, the use of an antigen index was described as proposed by Bos et al [29]. This attempted to correct for non-specific binding of antigens to antibody, and for interspecies cross-reactivity of the antibodies in the sandwich, by coating a plate with normal non-immune IgG from the same animal species, and running samples in parallel. No control virus or calibration curve was needed.

The final index was given by the formula

$$\frac{\text{O.D. of sample bound to anti-HSV1 IgG - Blank}}{\text{O.D. of sample bound to non-immune IgG - Blank}}$$

The reported positive results on human CSF's prompted a reproduction of this assay, to compare with the standard assay.

A duplicate plate was run identically to the assay described in section i), with the modification of a mouse normal IgG diluted to the same IgG concentration as the monoclonal Q2 (1:500) being used as the 'captor'. As a total of 200 ul of CSF was needed for each complete assay, only 4 of the HSVE samples could be run in this way.

C. Comparison of in-house assay with a commercial kit

Wellcome diagnostics had marketed an ELISA based HSV antigen detection kit, primarily for use in genital scrapings from suspected herpetic lesions. From discussion with the research team, anecdotal reports had been received of the success in childhood HSVE CSF analysis on an individual basis, and one trial report in adults using a modification of the kit has appeared in the literature, described in chapter 2 [6]. The nature of the antibody specificity of the kit was not disclosed, but appeared to be anti-glycoprotein C antibodies, common to HSV1 and 2. The positive control in the kit was said to be inactivated whole virus preparation, but Wellcome also gifted positive and negative controls which contained all the relevant antigens for both assays.

The control samples, both gifted and kit provided, were used neat and 0.1% Tween 20 treated in the in-house standard assay to ensure a positive response. Since the effect of acid neutralisation on the commercial kit could not be assessed, 4 HSVE CSF samples positive for free antigen and 6 control specimens were run in the commercial assay as per manufacturers instructions. Controls comprised one patient each with SSPE, neurosarcoid, Lyme disease and dementia and 2 patients with MS. A standard HSV Kos calibration curve was also included to compare the sensitivities of the assays.

2. Results

A. Sandwich capture assay

i) The standard assay

Figure 28 shows the calibration curve, corrected for control values, for HSV antigen detection. Plate to plate variability in blank background counts was moderate, at 0.046 to 0.086 O.D. units, but the corrected calibration curves were all identical in slope and sensitivity. This background variability depended on the day of the run, as duplicates on the same day had the same background. It was probably a result of the variability in the ambient temperature, since the assays were all run in the summer, and the room temperature could not be accurately controlled. Standardisation was also confirmed by the positive control run on each plate, which showed a constant signal:noise ratio of 1.6-1.8 on each plate, but paucity of specimen prevented any statistical analysis being performed on this.

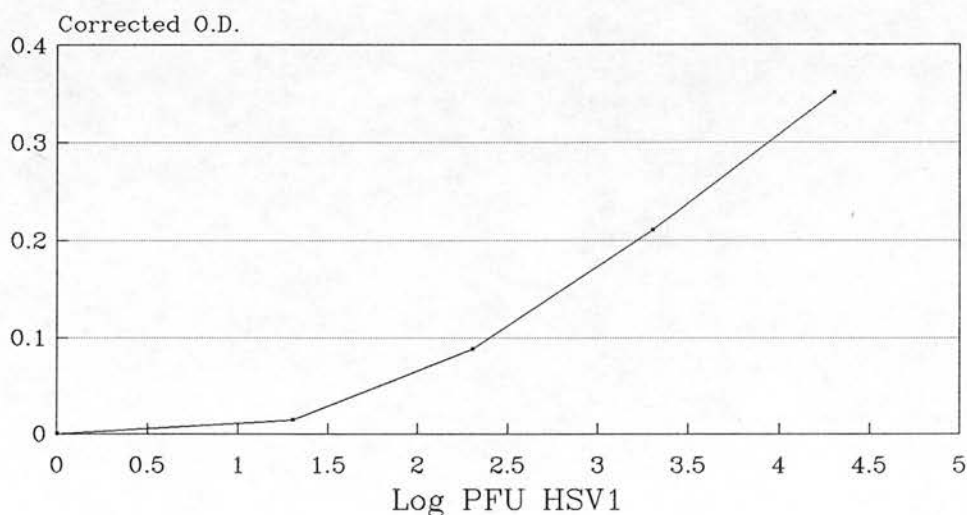


Figure 28 : Calibration curve for HSV1 alkaline nuclease sandwich assay

Table 29 shows the PFU values calculated from the calibration curves, for the HSVE and control samples with and without acid treatment. The control patients were grouped into diagnostic categories, including suspected herpetic infections.

All 12 CSF samples from the 9 confirmed cases of HSVE were positive for antigen, either treated with acid, untreated or both. Values ranged from 44 to 946 PFU in each 50 ul sample. Acid dissociation in 7 of the 12 CSF's resulted in clear increases in O.D. values, indicating the presence of immune complexed antigens. Indeed, in 5 of these CSF's, there was only complexed antigen detected.

When the CSF's were ordered on the basis of time of LP from day of onset of clinical symptoms (Table 30), it was found that before day 13 only free antigen (or predominantly free antigen) was present, by day 13 immune complexes were forming, and after day 14, save for two samples, there was no free antigen detected, all antigen being bound. Free antigen was detected on day 1 of the clinical illness in one patient with such an early CSF from LP. One patient with a prolonged prodrome to her illness had a CSF sample on day 47 of the initial illness showing only complexed antigen, then after a sudden acute deterioration a CSF taken after 7 days showed the presence of free antigen, but inexplicably no bound. A second patient had been initially treated abroad with a vidarabine to which he had initially responded. When transferred to the UK after 8 weeks, because of rapid deterioration, the CSF contained free and bound antigen, and subsequently acyclovir was administered, with unfortunately only a modest response.

Diagnosis	PFU counts		Local synthesis specific Ab
	Free	Complex	
HSVE			
1. CL a	0	72	-ve
b	0	64	-ve
2. ED	250	946	LS
3. TJ	448	472	LS
4. CH a	40	0	LEAK
b	0	70	LS
5. SP	0	44	-ve
6. NK	46	0	-ve
7. JM	46	0	-ve
8. PB a	0	40	LS
b	142	0	LS
9. MH	438	0	LS
INFECTIONS			
Herpes myelitis	>40000	>40000	LS
SSPE	90	502	-ve
SSPE	0	564	-ve
TSP	0	0	
TSP	0	0	
Neurosyphilis	0	0	
TBM + ?HSVE	0	112	LS
Lyme disease	0	0	
GBS	0	64	
GBS	0	0	
DEMYELINATION			
MS	0	0	
MS	0	0	
MS	0	0	
MS	0	0	
MS	0	0	
MS	0	0	
INFLAMMATORY			
Stiff Man	0	0	
Neurosarcoid	0	0	
OTHER CNS DIS			
Tumour	0	0	
Myelopathy	0	0	
Dementia	0	0	
Neurofibroma	0	0	
Paraprotein	0	0	
Dementia	0	0	
Dementia	0	0	
NON-CNS DIS			
Headache	0	0	
Sciatica	0	0	
Herpes labialis	0	0	LEAK
Lumbar disc	0	0	
AA subluxation	0	0	
?HSV INFECTION			
Encephalitis	662	264	
Encephalitis	0	0	
Encephalitis	0	380	
Encephalitis	752	598	
Myelo-radiculopathy	0	84	

For abbreviations see text

TABLE 29: Results of sandwich ELISA assay for HSV alkaline nuclease in 9 HSVE patients and 35 controls.

Table 30 also shows the results of CSF analysis for locally synthesised oligoclonal HSV1 specific IgG, local synthesis being defined as the presence of three or more clones of IgG in the CSF not present in matching serum. Only 6 of the 12 CSF's had locally synthesised anti-HSV1 IgG, with 1 CSF showing an identical serum and CSF pattern, a leakage pattern, indicative of passive transfer of IgG from serum. The earliest positive response was on day 4 of the illness, and after day 14 only 2 of 5 CSF's were positive.

9 of the 35 non definite HSVE patients were positive for HSV alkaline nuclease, 5 of these were from patients with definite infective pathologies: one was a herpes simplex myelitis, one a case of TBM with suspected coincidental HSV reactivation, one a post-HSV GBS and 2 were patients with subacute sclerosing panencephalitis (SSPE).

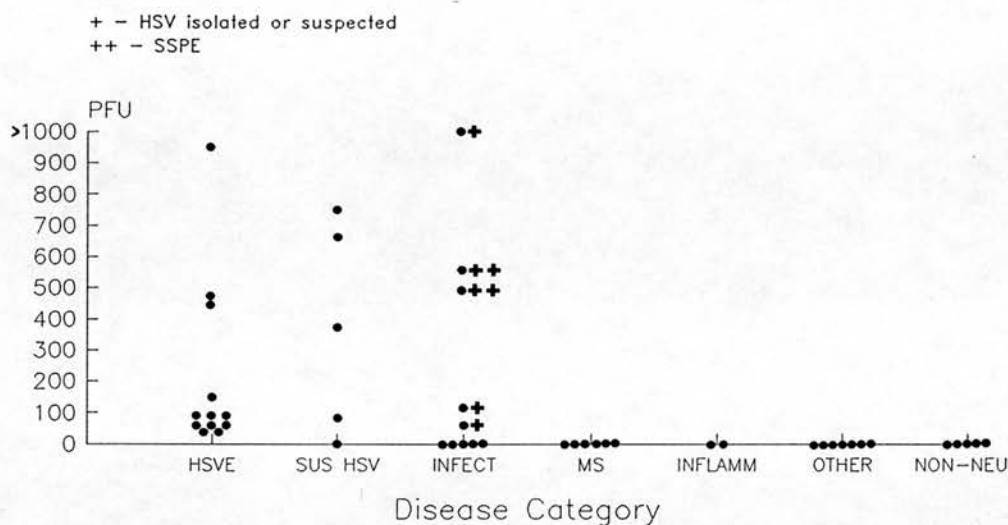
The remaining 4 patients had pathologies where the differential diagnosis included HSV as a causative agent: 2 were brain stem encephalitides, 1 a generalised encephalitis, and 1 a myeloradiculopathy. None had positive viral isolation from CSF, paired CSF and serum titres for HSV were not performed, but 3 were acyclovir treated with response.

Time from clinical onset (days)	Antigen Present		CSF Antibody Response
	Free	Complexed	
1	+	-	-ve
3a	+	-	-ve
4	+	-	-ve
4	+	-	+ve
13	+	+	+ve
14a	-	+	+ve
17	-	+	-ve
21b*	-	+	-ve
28b*	-	+	-ve
47c	-	+	+ve
54c	+	-	+ve
56	+	+	+ve

a,b,c - serial CSFs from individual patients

* - HSV1 isolated from CSF

TABLE 30: Duration of clinical illness and result of CSF antigen detection in patients with HSVE



For abbreviations see text and Tables
 Figure 29 : Alkaline nuclease assay results for disease categories

Figure 29 shows the results plotted in the form of a distribution histogram, according to category. In categories where infective causes could be excluded, none of the samples produced a positive result. One control patient had herpes labialis, but was negative for alkaline nuclease in CSF, suggesting that the antigen did not cross the intact blood CSF barrier, and thus excluding serum as a possible source of false positives.

ii) The HSV1 antigen Index

Blank values for the test plate were all higher than the blank values for the control plate by a factor of 1.33. This corroborated the findings of Bos et al [29]. Using the value of 1.5 as the cut-off recommended for the HSV standard to control standard, the minimum acceptable value for a positive result was 1.5×1.33 , or 1.995, which was rounded up to

2.0 for convenience. Individual samples were duplicated up to 3 times if volume permitted. For results that were at or below the blank value, an arbitrary value of 0.001 had to be allotted to allow an index to be calculated.

Table 31 shows the results of the antigen index so obtained. Some samples had also been assayed by the standard ELISA, and those results were included. Only 2 of the 4 HSVE CSF samples from 3 patients were positive by the index; one agreed with only finding complexed antigen, one agreed with finding free antigen but failed to find complexed, and 2 failed to find complexed antigen.

Of the 37 non definite HSVE and control patients tested, there were 6 positive results. Two were from cases of SSPE, both of whom had been positive by the standard ELISA, and one was a suspected case of HSVE, which had not been previously assayed. Two were from other infectious diseases, namely Lyme disease and tropical spastic paraparesis (TSP), and one from a patient with MS, all of which had been negative by standard ELISA. The remaining 31 samples were negative: 12 of these had been tested by the standard ELISA, of which 4 had been positive.

Diagnosis	Antigen Index		ELISA result	
	Free	Complex	Free	Complex
HSVE				
1. CL a	1.43	1.46	-	+
b	1.24	1.52	-	+
3. TJ	3.46	1.50	+	+
4. CH b	1.69	2.59	-	+
INFECTIONS				
SSPE	2.42	-	-	+
SSPE	1.34	2.00	+	+
Lyme disease	0.40	2.11	-	-
Lyme disease	1.32	1.38		
HSV1 myelitis	1.44	1.50	+	+
TSP	1.21	1.20	-	-
TSP	1.25	2.27	-	-
GBS	0.20	0.56	-	-
GBS	1.26	1.07	-	+
TBM	1.00	-		
TBM	1.00	0.91		
Neurosyphilis	0.64	1.17		
Meningitis	1.00	0.97		
Meningitis	1.06	1.11		
Malaria	1.00	1.03		
Varicella enc.	0.03	0.43		
Mumps enceph.	1.00	0.61		
Toxoplasmosis	0.80	0.90		
Brucellosis	1.00	0.84		
DEMYELINATION				
MS	1.08	1.67	-	-
MS	1.17	5.21	-	-
INFLAMMATORY				
Takayasu's	1.00	1.00		
Wegeners	0.62	0.82		
Neurosarcoid	1.00	1.13		
Neurosarcoid	1.37	1.85	-	-
OTHER CNS DIS				
Tumour	1.48	1.73	-	-
Dementia	1.20	1.31	-	-
Dementia	1.09	1.23	-	-
Myelopathy	1.15	1.21	-	-
?HSV INFECTION				
Encephalitis	1.32	2.04		
Encephalitis	1.21	1.75		
Encephalitis	0.93	0.53		
Encephalitis	0.46	0.59		
Encephalitis	1.00	0.79		
Encephalitis	1.21	1.33	-	-
Encephalitis	1.27	1.30	-	+
Encephalitis	1.40	1.14	+	-

For abbreviations see text

TABLE 31: Results of antigen index for HSV alkaline nuclease in 3 patients with HSVE and 37 controls.

B. Comparison of the in-house assay with a commercial kit

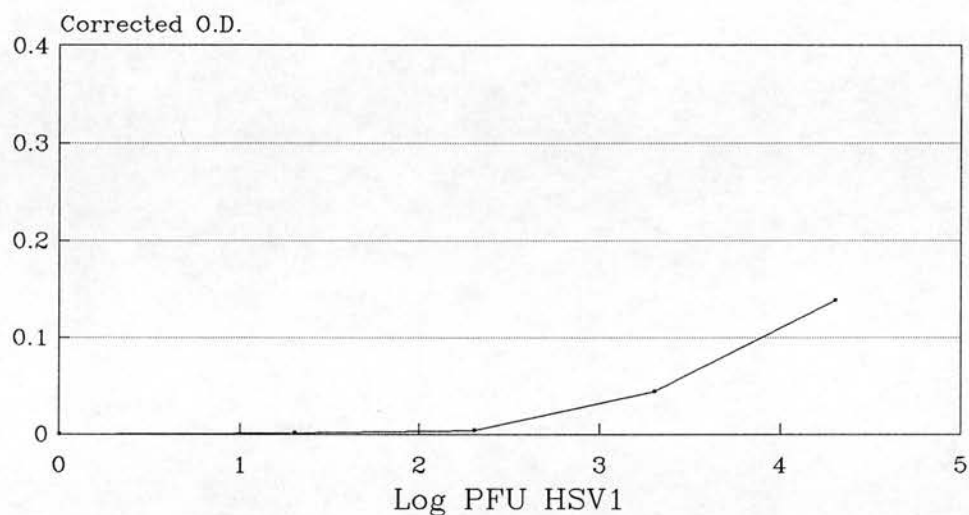


Figure 30 : Calibration curve for HSV1 using commercial Wellcozyme assay

The antigen content of the kit and gifted positive controls was estimated at 100,000 and 20,000 PFU respectively. Figure 30 shows that the standard curve obtained using the commercial kit was less sensitive than the alkaline nuclease based ELISA with at best about 1500 PFU detection limit, a factor of 70-fold less sensitive. None of the HSVE patients were positive using the kit, using the criteria defined by the manufacturers, and none of the controls were positive, even the SSPE control, presumably because of this reduced sensitivity.

3. Discussion

In the standard ELISA assay, acid dissociation, when used on control samples that were clear negatives, made no consistent difference to the values, with equal numbers showing a slight increase or decrease in O.D., and never creating a positive result from a negative result. Changes in O.D. were <10%, suggesting that there were negligible artefactual effects on the ELISA assay component reagents. When an untreated CSF sample was positive, either HSVE or control, acid dissociation often led to a reduction in O.D. values (5 of 7 CSF's from confirmed HSVE patients, 3 of 4 from non-HSVE patients), and in some cases rendered the count down to background. This may have been due to poor neutralisation of acid with alkali, resulting in some continued chaotropic effect, or to reduction in the immunological reactivity of the antigen in the CSF. This latter explanation would seem unlikely considering the apparent lack of effect seen in the preliminary experiments, but in any case, the end result made it difficult to predict the 'true' value for acid-dissociated antigen, which must have been higher than the calculated value and also resulted in some cases with small amounts of only bound antigen being undetectable.

There was a clear association between the development of a locally synthesised IgG immune response and the presence of free or bound antigen. Of course, these were not serial punctures in an individual patient, but a montage from many. The development of the response is still of interest since it seems to parallel the early absence of any specific IgG leaving only free antigen, then the development of specific IgG, and finally the immune complex formation. The 3 post 14 day negative CSF's came from 2 patients, both of whom produced a late antibody response after these CSF samples, and one of these had HSV1 isolated from the CSF, illustrating that individual patients could produce widely differing immunological responses. This reinforces the need to be cautious about interpretation of combined data.

Amongst the control patients studied by the ELISA, the greatest response from any sample was in the CSF from the case of herpes simplex myelitis, and probably reflected the proximity of the needle to the pathology. The results for SSPE were unexpected, but were unlikely to represent cross reactivity since measles virus gave counts at background level in the assay. The possibility of reactivation of latent HSV secondary to the measles infection is likely, given that reactivation is stimulated by neurological insults, as described in chapter 4. The result for post genital HSV GBS is of interest in that it suggests either virus gains access to the CNS via a disrupted blood CSF barrier, or via retrograde transportation of virions, resulting in disruption. Whether an underlying myelitis was present would be impossible to assess in the presence of severe peripheral nerve involvement.

The assay thus confirmed the clinical and laboratory diagnosis of HSVE by the detection of HSV specific alkaline nuclease in all 9 patients, and in all CSF samples studied. In addition in 2 patients with other HSV CNS infections, in 5 out of 6 samples with suspected HSV pathologies, and both patients with SSPE, alkaline nuclease was also detected. No control patient with a non-infective aetiology produced a positive result. The specificity of the assay was thus 100% and the sensitivity in definite cases 100%.

The index, in contrast, had major drawbacks, mainly in the mathematical interpretation of the results. Most of the problems lay in calculating the index from the blank O.D. value. Although the difference between the control and test plate blanks was a constant at 1.33, many CSF samples produced results at or below the blank on the control plate, presumably as a result of the presence of other proteins in the CSF acting as blocking agents, or binding to cross reacting antibodies. Calculations could not cope with negative values, so an arbitrary value of 0.001 was assigned, which although in the main did not result in unreasonable indices, in some cases resulted in indices far higher than were consistent with the actual O.D. values. All other methods of correcting for background produced wildly varying results for some samples, so were discarded, for

example using the lowest test value in each group as the blank, calculating an uncorrected ratio or calculating an average blank value based on all test values below the 'real' blank. There was also unacceptable variability in the duplicated results, probably because 4 variables were being incorporated into the calculations instead of 2, so only consistently positive or negative samples could have average values calculated with any degree of certainty.

Overall, the index was not as robust or reproducible as the standard ELISA. In addition it was expensive in sample volume required (200 ul per assay), and the results obtained neither as internally consistent as the standard ELISA, nor consistent with the known diagnoses, especially with the detection of known positive samples.

Comparing the two methods in the definite HSVE group, the index performed relatively poorly, detecting a positive index in only 3 of the 4 specimens positive by the ELISA assay. Comparing the non definite HSVE and control patients, there was agreement on 8 results (all negative), partial agreement on 2 (SSPE), but complete disagreement on 7 (3 positive by the index, 4 positive by the standard ELISA) of which 5 were fundamental disagreements on certain diagnoses. The index thus had a false positive rate compared to the ELISA of 3 out of 17 (18%), and a false negative rate of 4 out of 17 (24%).

SECTION 3

DISCUSSION

Chemiluminescence has been hailed as a major advance in assay systems, with theoretical sensitivities of detection of chemiluminescent products approaching 100 molecules. These specialist studies have not however translated so effectively the theoretical laboratories into the routine chemistry laboratories, with bioassay responses more in line with conventional techniques. There are a wide variety of chemiluminescence generating systems, of which only two were used in this work, both having been extensively evaluated by others.

Chemiluminescence is attractive because of the relative ease with which reagents can be manufactured, and the assay times which are similar to conventional ELISA. Light output can be measured using elaborate equipment such as photo-multipliers, or simply using photographic film. The relative disadvantages are the multiplicity of extraneous factors that can interfere with chemiluminescence such as proteins, detergents, chaotropic agents, miscellaneous chemicals, temperature, light etc., and the cumbersome recording of light output detection systems, where only one sample at a time can be assayed, although this reflects the limits of available technology and may change.

In this study with chemiluminescence, it initially appeared that isoluminol based chemiluminescence was more sensitive than luminol based assays, which prompted a great deal of work conjugating antibodies, assessing specific activities, affinity purifying the conjugates and attempting assays on a variety of systems. The problems of conjugation however were the loss of biological activity which the label induced, and the difficulty in affinity purification of labelled antibody without destroying the labels' chemiluminescent activity. The loss of biological activity was probably related to mechanical interference, since labelling was a random event, and thus as likely to occur on the Fab region of the immunoglobulin molecule as the Fc region. Attempts to 'cover' label by binding antibody to antigen fixed to a column, thus covering the Fab binding site, and then labelling so that only sites not involved in antigen binding were the only

ones available did not work, mainly because high affinity labelled antibody could not be eluted which retained chemiluminescent activity. Although NaSCN had the most inhibitory effect on chemiluminescence, lesser degrees were seen with pH, NaCl and detergent, and the inhibition was the same irrespective of the antibody labelled.

Subsequently, luminol chemiluminescence was re-investigated, particularly when the problems with the kinetics were discovered to be due to artefacts generated by free O^- radicals in the H_2O_2 from NaOH activity (despite this being recommended in the literature), and the resulting sensitivity with iodophenol enhancement was found to be far in excess of isoluminol. Another advantage was the use of commercial affinity purified HRP conjugated antibodies, which meant that background activity was considerably reduced. The increased sensitivity however was not always predictable. The molecular basis for iodophenol enhancement has not yet been elucidated, but enhancement does not result solely from action on the HRP molecule since colorimetric assays were not enhanced by iodophenol (data not shown). In the absence of HRP, iodophenol inhibited spontaneous luminol chemiluminescence, and when HRP concentrations were below a particular threshold value, iodophenol inhibited chemiluminescence. This limit of enhancement proved to be a major problem. Although in assays of sensitivity of detection of the HRP catalysed response, up to $1:50 \times 10^6$ dilutions of stock non-purified conjugates, this detection resulted from the unbound HRP molecules. When affinity purified conjugates were used the limit of detection was greatly reduced at around $1:10^5$. Iodophenol appeared to be acting as a stabiliser on both the HRP and the luminol molecules, but this was concentration dependent. This variability in the response was also reflected by the optimum iodophenol concentration required for the assay, which differed depending upon the concentration and type of HRP conjugate used.

Thus when calibration curves for HSV antigens with identical reagents were compared using colorimetric and chemiluminometric detection, there was no difference in the sensitivity, presumably because at the lower HRP concentrations iodophenol was unable

to enhance, although at higher concentrations there was a much greater signal generated for chemiluminescence enhanced by iodophenol. In practical terms, colorimetric analysis became the preferred method, mainly because of the ease of measurement, but also because of the problems above.

The best solid phase for the assay was the microtitre plate. Although sepharose offered the attraction of a larger surface area:volume for mixing and protein binding, the handling of the material was cumbersome and washing the gel was not only time consuming, but also less efficient because of the entrapment of small molecules within the lattices of the gel. Polystyrene balls offered a larger surface area than the microtitre well, and semi-automated ball and reaction tubes were readily available, but again the method was cumbersome, relatively large amounts of sample were needed to cover the balls, and reagents were unstable with time. Attempts to improve stability and sensitivity with PPL and protein A coating were only partially successful with stability improving but not the sensitivity. Although protein A had tremendous theoretical advantages as a surface binding agent, in practice optimising the coating was difficult, and blocking the unbound sites resulted in interference with the captor antibody coating. Sensitivity achieved by this assay was much better than for sepharose, but was still only at 1:100,000 dilution or 3×10^4 PFU. This may not have mattered when sensitivity was not important, but for the purposes of antigen detection, which was expected to be at the limits of sensitivity anyway this was unacceptable.

The use of microtitre plates is now widely established as a standard solid phase for ELISA techniques. The convenience of handling, washing and result gathering, especially with multi-channel pipetting and automated plate readers, makes it ideal for screening and optimising assays. The availability of microtitre plates with detachable wells meant that chemiluminescence could also be assessed using this solid phase. Plate to plate variation in results was far more acceptable, and background counts were the lowest of the three solid phases, resulting in consistent standard curves. There was some

day to day variability resulting from variations in the ambient temperature. Only small volumes of CSF were required (50 ul), and overall sensitivities achieved were the best of the solid phases.

Altogether a total of 10 antibodies were tried in various combinations in the sandwich assay: 2 were commercial, one polyclonal and one monoclonal, one was human IgG, 6 were high affinity rabbit polyclonals, all gifted, and one a mouse monoclonal also gifted. Of all the sandwich systems tried, it came as somewhat of a surprise to find that the best combination for the assay was to use a monoclonal captor and a polyclonal detector. One would have anticipated that by capturing with a polyclonal, the chances of capturing the epitope of interest would have been optimal, and the monoclonal as detector would have provided greater specificity. The fact that the reverse was true meant that the logic was incorrect. The polyclonal antibody must have occupied either the site necessary for the binding of the monoclonal, or adjacent sites resulting in an adverse influence on the binding. By capturing with the monoclonal, only one part of the molecule would have become inaccessible, leaving the other sites still available for the relevant antibodies to bind to.

The HSV alkaline nuclease turned out to be the best viral antigen to use as a marker of viral presence. Antibodies against non-envelope structural proteins (205/5), structural late proteins, including VP 13 (220/3), envelope glycoproteins (commercial monoclonal) and pan-proteins (R38/5, human IgG, commercial polyclonal, alpha-T1, alpha-T2) were all less effective. Alkaline nuclease is synthesised in large quantities in productive infection, and in the context of HSVE, where virus is released from necrotic tissue, of all the antigens produced it should be one of the easiest to detect. The humoral response to glycoproteins, being much more prominent in serum, may more effectively dispose of these antigens by immune complexing, thus reducing the sensitivity of any assay attempting to detect these. Although the alkaline nuclease of HSV1 and HSV2 have

common antigens, it is not important clinically to differentiate the two as treatment will not be influenced, and serological responses can be used in retrospect to distinguish one from the other.

The results of the assay run on patients CSF showed that free HSV antigen was detected from day 1 of the clinical illness. By day 13, antigen was becoming immune complexed, and after this time immune complexed antigen predominated, with only two patients showing free antigen, both of these undergoing acute deteriorations at the time. This paralleled the immune response in HSVE described in Chapter 1, where the earliest antibody production is IgM from days 3-4 and IgG from days 5-7, peaking at around day 10, and remaining elevated for months after the original illness [81, 188]. Thus, in the early phase, free antigen predominates until the immune response is switched on sufficiently to produce enough antibody to complex antigen by day 13, and then overwhelm the antigen thereafter. This would account for the findings reported by other workers, where antigen was only recoverable in the first 10-14 days, and thereafter could not be detected, whereas immune complexes became apparent after day 10. This may also explain the difficulties in culturing virus from CSF, despite overwhelming infection. The virus has already been immune complexed by the the time most attempts at viral isolation have been made, and only when the puncture is at a critical phase of sufficient viral release into the CSF with minimum antibody production will culture be possible.

Of course, result interpretation is severely limited by the lack of numbers, and one could argue that the pooling of data regarding time course of the response is not valid, since each individual will behave in a unique fashion. This would more likely result in negative correlations, rather than the positive findings here, even with the small number of data points. Two of the patients did behave in an atypical way in terms of the immune response, with delay of intrathecal specific IgG production until well after day 10. In practical terms, serial CSF samples on individual patients in this country are extremely unusual, as there is little ethical justification when a diagnosis has been made, treatment

instituted and a response obtained. It is only when the diagnosis is in doubt that 2 or more punctures will be performed. In three years at the laboratory where this study was performed, only one patient had 3 CSF samples during the course of the disease, and normally only 2 LP's were performed, unless a patient relapsed. One late relapsing case was included in this study, although only the CSF sample immediately after the onset of the relapse was available. During the relapse free antigen again appeared in the CSF.

The recovery of complexed antigen after 47 days from the CSF further fuels the debate as to the persistence of the specific IgG response in the CSF of patients who have recovered from HSVE. It has been suggested that this reflects a failure of immune surveillance mechanisms, with switching off after successful removal of antigen not taking place, and that the abnormal immune response may underlie why such patients are susceptible to CNS invasion in the first place. Others have suggested that persistence of specific antibody must reflect persistence of the offending antigen, and that those patients must have continual antigen presentation to sustain such a response. This may mean persistence of active viral replication and shedding from an individual site within the CNS, that does not manifest itself clinically, or continued antigen presentation of the surface of lymphocytes. The results from this single patient would support this latter view of antigen persistence, and if accepted as such means that the current regimes for the treatment of HSVE may be inadequate. This possibility has been raised in recent reports documenting the failure of the current 10 day course of Acyclovir, with relapses shortly after discontinuation of the drug, and suggests that much longer courses are necessary. In our group of 9 patients, all were treated with acyclovir for the full 10 day course, yet 3 relapsed and responded to a repeat course, and one despite initial response to cytarabine relapsed and made some response to acyclovir. Only by studying CSF taken from patients without clinical relapse at times distant from the initial event will this question of persistence be resolved. Since ethical approval is likely to be difficult to come by for such a study in the UK, this issue will likely remain the subject of conjecture. One way or the other, it may be prudent to revise the schedule for acyclovir

administration, erring on the side of caution in assuming current undertreatment, especially in view of the low toxicity of the drug, and by the 10th day of the illness the diagnosis should be confirmed.

The other problem in the collection of specimens which was apparent from this study was the poor quality of the clinical and laboratory diagnosis. More than 20 patients were referred with the diagnosis of HSVE during the period of the study. The majority had only a single CSF sample taken, and the diagnosis based solely on a rising titre of serum antibodies, or even static low titres! All patients were treated with acyclovir, so clinical improvement in some, which was attributed to therapy, could equally have been coincidental, and thus should not have been taken as evidence of HSV infection. No patient referred had brain biopsy performed, and in 2 cases retrospectively diagnosed as HSVE from post mortem analysis, no CSF was saved. The cases may have been genuine HSVE, but the lack of definitive virological or immunological proof meant that these had to be excluded. This problem with inadequate data may partly explain the shortfall in the cases documented by the PHLS.

The use of acid dissociation of immune complexes has been shown to be feasible and very effective, as with HIV p24 antigen detection. This study showed that the same technique could be applied to HSV1 immune complexes, with successful dissociation and detection in the sandwich assay. However there did appear to be a reduction in the capture of antigen after acid treatment, so that immune complexed results represented an underestimation of the amounts present. This may have been due to technical failure, or may indicate the fragility or susceptibility of the alkaline nuclease to hydrolysis. Lower concentrations of acid and alkali were not used, mainly because of the unexpected nature of the result, considering the early studies gave no indication of any effect on the antigen, and little CSF was available to try variations on patients' samples. Whether any

other means of disrupting immune complexes would be less damaging to antigen is uncertain, and may not be practicable since some means of neutralising the effect must be available to use a capture antibody system.

The best means of interpreting the results was by a straight plot of the corrected O.D. against a standard calibration curve. This gave reproducible results, even when the background count varied as described. The alternative method, proposed by Bos et al, was found to be poorly reproducible and results inconsistent with the standard assay, mainly because of the mathematics involved in the calculation, and possibly also because of the assumption that a polyclonal control antibody would behave as an effective control for a monoclonal antibody. More importantly, the assay resulted in false positive results in the control patients who did not have herpetic infections, as well as false negative results in the definite HSVE patients. For these reasons, and also because of the requirement for double the volume of CSF, the method was completely unreliable, and so was discarded.

In contrast, the standard assay involving a calibration curve produced no positive results in the control patients without suspected herpetic aetiologies. In the 5 patients with suspected herpetic CNS infections, 4 proved positive by the assay, and all 3 control patients with non primary HSVE herpetic infections were positive. The most unexpected finding was the positive results in the 2 SSPE patients, which suggests that intercurrent disease may reactivate latent HSV, or be permissible for primary invasion of the CNS by HSV. At least some component of what is a universally fatal disorder may thus be treatable, and although this is unlikely to affect the outcome, since the reason for reactivation of measles and the relentless progression is unknown, and co-factors may be important (as with HIV infection), therapy of any treatable component may be worthwhile.

The importance of being able to detect antigen as early as possible has already been stressed in the introductory chapters. Since this was accomplished in this study in every CSF from all patients, and from day 1, treatment of suspected cases can be initiated immediately and a result for antigen detection be available by the following working day. The patients reported here all had unequivocal HSVE - there are likely to be a larger number of patients with HSVE in whom the diagnosis is never made, because of 'atypical' clinical signs, insufficient laboratory data in clinically likely cases or mild forms that are overlooked. The application of this or similar antigen detection techniques to these forms would be of value. In some ways, these groups would be the more important to study, since these are likely to remain untreated, especially if conventional techniques such as CT or EEG are negative, and morbidity advanced before HSVE is considered. With the increased numbers of patients immunosuppressed by HIV, the presentation of HSVE may become more atypical, and perhaps these should be also be screened.

In conclusion, although only one of the two aims of this work were fulfilled, it was the more important of the two, that is, the development of a method of detection of HSV specific antigen in the CSF of patients with HSVE. Further work is needed on a larger number of patients to establish the limits of the system, before it can be applied clinically. Even if the method does not prove to be as robust as it seems so far, so that it cannot in its present form be used for large scale screening, the findings of the assay are still relevant. Antigen is present from day 1 of the clinical illness, and is recoverable using a relatively simple assay system. This in itself should prompt the search for better antibody combinations and detector systems.

APPENDICES

APPENDIX 1 : Biuret method for total protein estimation [1]

4.5 g of potassium sodium tartrate is dissolved in 40 ml 0.2 M NaOH. 1.5 g copper sulphate is added, with continual stirring until completely dissolved, followed by 0.5 g potassium iodide, and the whole made up to 100 ml with 0.2 M NaOH. For use, the solution is diluted 1:5 with 0.2 M NaOH containing 5 g/l potassium iodide.

50 ul of the test protein is mixed with 2.5 ml of the working strength biuret reagent to produce an instant change in blue colour. The reaction is allowed to go to completion by leaving for 15 min at RT. The reaction mix is then read at 555 nm in a spectrophotometer, using biuret solution alone as the blank. The calibrant protein is treated identically to produce a standard curve.

APPENDIX 2 : Purification of IgG by Rivanol extraction [104]

500 ul of the antiserum to be purified is mixed with 345 ul distilled water, then continually agitated whilst 325 ul of 3% Rivanol (pH 7.5) is added slowly. An orange/yellow precipitate forms, which is discarded after centrifugation - this contains all proteins except transferrin and immunoglobulins. To the supernatant is added solid NaCl with continual mixing until the mixture becomes a creamy 'lemon curd' - this removes rivanol. After centrifugation the precipitate is discarded. An equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ is added to the supernatant with mixing, and after centrifugation the supernatant, containing transferrin which does not precipitate, is discarded. The precipitated immunoglobulin is washed by adding 1 volume 50% $(\text{NH}_4)_2\text{SO}_4$, centrifuging and discarding the supernatant. The pellet is then resuspended back to the original volume in 0.15 M NaCl, and then dialysed in visking tubing for 18 hr at RT against 2 litres 0.15 M NaCl. The purified immunoglobulin is stored at 4°C. Purification is checked by standard agarose electrophoresis on ultrathin gel of pre- and post-purification serum and staining with Coomassie blue.

APPENDIX 3 : Clinical details of patients with HSVE

1) NK, 68yrs, Female, CSF day 1.

An acute confusional state, pyrexia, hemiparesis. CSF contained 8 WBC, 0 RBC. EEG demonstrated a focal temporal lobe abnormality. Paired serum and CSF showed > 4-fold rise in titres with local synthesis in the CSF. Acyclovir administration led to complete recovery, but relapsed 2 weeks after completion of therapy, and responded to a second course.

2) SP, 26yrs, Female, CSF day 17.

Two week history of focal fitting, with confusion and behavioural disturbance. EEG showed a temporal lobe focus, CT was normal. Genital herpes was found, HSV1 was cultured from the scrapings. CSF contained 16 WBC, 4 RBC. Paired serum and CSF showed no serum response, but a > 4-fold elevation in CSF titres. Acyclovir was administered after the first LP, but the patient was left with psychiatric disturbance and memory loss.

3) CH, 37yrs, Female, CSF days 3, 14.

Three day history of aphasia, hemiparesis and pyrexia. EEG showed a focal temporal abnormality, the CT was normal. CSF contained 61 WBC (34 mononuclear, 27 polymorphs), 18 RBC. The first CSF and serum pair showed local synthesis of specific antibody. Acyclovir treatment resulted in complete recovery.

4) TJ, 65yrs, Male, CSF day 13.

Thirteen day history of headache, malaise and 1 day collapse. EEG showed a temporal lobe focus and focal periodic complexes, the CT showed a temporal lobe abnormality. Paired CSF and serum showed local synthesis of specific antibody. Acyclovir therapy resulted in an initial deterioration followed by recovery to normality.

5) JM, 33yrs, Male, CSF day 4.

Four day history of malaise, headache, pyrexia and behavioural change. After admission the patient developed temporal lobe epilepsy and autonomic failure. CSF contained 72 WBC (99% lymphocytes). First paired CSF and serum samples were negative for HSV1 antibody, the second set were positive with intrathecal synthesis. The CT scan showed an ill-defined abnormal temporal lobe area, the EEG a clear temporal lobe focus. Following acyclovir therapy there was recovery, but the patient was left with a global memory impairment.

6) ED, 32yrs, Male, CSF day 56.

Admitted to a hospital abroad with fever, headache, confusion, meningism and epilepsy, but no focal neurological signs. The CT scan was normal, the EEG showed diffuse abnormalities without focal features. CSF contained 880 WBC (85% lymphocytes). After treatment with cytarabine there was initial response, but six weeks later he relapsed, with aggression, hyperphagia, hypersexuality (reminiscent of the Kluver-Bucy syndrome), then progressing to coma. On transfer to the U.K. his EEG was still generally abnormal, the CSF contained 13 WBC (97% lymphocytes), and there was local synthesis of HSV1 specific IgG. The MRI demonstrated bilateral temporal lobe abnormalities. A repeat course of acyclovir resulted in an improvement in the conscious state and the EEG, but the psychiatric problems persisted.

7) MH, 34yrs, Male, CSF day 4.

Four day history of malaise, drowsiness, dysphasia, arm weakness and sensory disturbance with pyrexia. CSF showed an elevated total protein only. CSF showed intrathecal synthesis of specific antibody. CT scan was normal, the EEG showed a temporal lobe focus. Acyclovir administration was followed by an initial deterioration, then recovery. Three weeks later the patient relapsed, a further course of acyclovir was given with recovery, but the patient was left with mild behavioural abnormalities.

8) PB, 48 yrs, Female, CSF days 47, 54.

A six week history of slowly deteriorating intellect and headache, followed by a sudden 5 day worsening with confusion. Both CSF samples showed local synthesis of specific antibody. Following acyclovir treatment, there was complete recovery.

9) CL, 42 yrs, Female, CSF days 21, 28.

Following headache, malaise, fever, sore throat and nausea the patient presented with three weeks of progressive tetraparesis and sensory loss, with brain stem and autonomic dysfunction. Recent herpes labialis infection had occurred. CT brain was normal, but brain stem was not specifically examined, and the EEG was also normal. CSF contained 28 then 35 WBC (95% lymphocytes), but there was no specific antibody response in either of the CSF's available, although later CSF did show specific IgG. HSV1 was isolated from the first CSF before therapy. Acyclovir was followed by clinical improvement, but virus could not be isolated from subsequent CSF samples.

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